The Effects of Cannabinoids on IL-1β Induced Catabolic Pathways in Osteoarthritic Chondrocytes

Sara Louise Dunn

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Abstract

A key feature of osteoarthritis (OA) is the loss of articular cartilage. Cartilage breakdown is mediated by complex interactions of proinflammatory cytokines such as interleukin 1β (IL-1β) and proteases including matrix metalloproteinases (MMPs).

Cannabinoids have been shown to reduce joint damage in animal models of arthritis and have also been shown to prevent IL-1 induced matrix breakdown of collagens and proteoglycans, suggesting a chondroprotective effect of these compounds. Cannabinoids mediate their effects via putative cannabinoid receptors and activation of these receptors has been shown to display anti-inflammatory activities and inhibition of destructive factors in human OA chondrocytes and synovial fibroblasts.

This thesis demonstrates that the synthetic cannabinoid WIN-55 inhibits the expression of ECM degrading enzymes MMP-3 and MMP-13 both at the mRNA and protein level in human OA chondrocytes. WIN-55 also decreased the expression of MMP inhibitors: tissue inhibitors of matrix metalloproteinases -1 and -2 (TIMP-1 and -2). WIN-55 inhibited IL-1β induced signalling pathways including ERK1/ERK2, c-Jun and IκB but not p38, these findings suggest a possible mechanism via which WIN-55 decreases the expression MMPs.

The classical cannabinoid receptors cannabinoid receptors 1 and 2 (CB1 and CB2), G protein-coupled receptor 55 (GPR55), G protein-coupled receptor 18 (GPR18), transient receptor potential vanilloid 1 (TRPV1) and peroxisome proliferator activated receptor alpha, delta and gamma (PPARα, δ and γ) were expressed by OA chondrocytes and osteocytes in the underlying bone, with a decrease in GPR18, TPRV1 and PPARγ in specific zones of the osteochondral compartment associated with an increase in grade of degeneration.

Selective cannabinoid receptor agonists were used to determine the receptor(s) via which WIN-55 may mediate its effects in human OA chondrocytes. Agonists for CB1, CB2 and PPARα, δ and γ used in combination inhibited IL-1β induced MMP-3 or -13 mRNA expression, suggesting that WIN-55 may mediate its effects via activation of multiple cannabinoid receptors or via a novel cannabinoid receptor.

Pro-inflammatory cytokine IL-1β is a well-known mediator of cartilage degradation, thus the inhibition of IL-1β induced expression of MMPs and intracellular signalling pathways shown here by WIN-55 suggests that cannabinoids could provide a model for the development of novel therapeutic agents for arthritis via preventing cartilage degradation.

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Abbreviations

ABC	Avidin/Biotin complex
AC	Adenylyl cyclase
ACEA	Arachidonyl-2'-chloroethylamide
ADAMTSs	A disintegrin and metalloproteinase with thrombospondin motifs
AEA	Anandamide (arachidonoylethanolamide)
AGEs	Advanced glycation end products
AIA	Adjuvant-induced arthritis
AJA	Ajulemic acid
AP	Alkaline phosphatase
APMA	Aminophenylmercuric acetate
AR	Antigen retrieval
AP-1	Activator protein 1
BDNF	Brain-derived neurotrophic factor
BMP-2	Bone morphogenetic factor protein 2
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
cAMP	cyclic adenosine 3', 5'-monophosphate
CB1	Cannabinoid receptor 1
CB2	Cannabinoid receptor 2
CBD	Cannabidiol
CBG	Cannabigerol
CBN	Cannabinol
CCL5	CC-chemokine ligand 5
cDNA	Complementary DNA
CGRP	Calcitonin gene related peptide
CNS	Central nervous system
COMP	Cartilage oligomeric matrix protein
COX	Cyclooxygenases
CXCR1	IL-8 receptor
Δ^8-THC	Δ^8-Tetrahydrocannabinol
Δ^9-THC	Δ^9-Tetrahydrocannabinol
DAB	3,3'-diaminobenzidine tetrahydrochloride
DAPI	4',6-diamidino-2-phenylindole

DMAODs	Disease modifying anti osteoarthritic drugs
DMARDs	Disease modifying anti-rheumatic drugs
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
dNTPs	Deoxyribonucleoside triphosphates
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinases
Ets	Erythroblastosis twenty-six
FAAH	Fatty acid amide hydrolase
FABP	Fatty acid binding proteins
FBS	Fetal bovine serum
GADD45β	DNA damage 45 beta
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPCR	G protein-couple receptor
GPR55	G protein-couple receptor-55
GPR18	G protein-couple receptor-18
GRO	Growth-related oncogene
HA	Hyaluronic acid
H&E	Haematoxylin and Eosin
HIF1α	Hypoxia-inducible factor-1-alpha
HRP	Horseradish peroxidase
HU210	(-)-11-hydroxy-Δ^8-THC-demethlheptyle
ICAM-1	Intercellular adhesion molecule 1
ICC	Immunocytochemistry
IgG	Immunoglobulin
IHC	Immunohistochemistry
IκB	Inhibitory kappa B
IKK	I kappa B kinase
IHC	Immunohistochemistry
IFNβ/γ	Interferon beta/gamma
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6

IL-8	Interleukin-8
IL-15	Interleukin-15
IL-17	Interleukin-17
IL-18	Interleukin-18
IL-19	Interleukin-21
IL4R	IL-4 receptor alpha chain
IL-1R1	IL-1 receptor type I
IL-1RAcP	IL-1 receptor accessory protein
IL-1Ra	IL-1 receptor antagonist
IMS	Industrial methylated spirits
iNOS	Nitric oxide synthase
IRAK1	Interleukin-1 receptor associated kinase 1
JAK/STAT	Janus kinases and signal transducer and activator of transcription
JNK	Jun N-terminal kinase
LEA	Linoleoylethanolamide
LPI	Lysophosphatidylinositol
LPS	Lipopolysaccharides
MAGL	Monoacylglycerol lipase
MAPKs	Mitogen activated protein kinases
MAPKKKs	MAPK kinases kinases
Mca	Methyl cumaryl amide
MCP	Monocyte chemotactic protein
miRNAs	microRNAs
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
MT	Masson Trichrome
MT1	Membrane type I
NaCl	Sodium chloride
NADA	N-arachidonoyl dopamine
NAGly	N-Arachidonoylglycine
NFκB	Nuclear factor kappa B
NGF	Nerve growth factor
NIK	NFκB-inducing kinase
NK1R	Neurokinin-1 receptor

NO	Nitric oxide
NT	Neurotrophin
OA	Osteoarthritis
OEA	Oleoyl ethanolamide
OLDA	N-oleoyl dopamine
OSM	Oncostation-M
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEA	Palmitoyl ethanolamide
PGE2	Prostaglandin E_2
PKA	Cyclic AMP-protein kinase A
PPARα/δ/γ	Peroxisome-proliferator activated receptor alpha/delta/gamma
PPII	Polyproline II type
R(+)WIN 55, 212-2	WIN-55
RA	Rheumatoid arthritis
RANKL	Receptor activator of nuclear factor kappa-B ligand
RFU	Relative fluorescence units
RNA	Ribonucleic acid
RT	Reverse transcription
RTX	Resiniferatoxin
RXR	Retinoid X-receptor
sdH_2O	Sterile deionised water
TACE/ADAM-17	Tumour necrosis factor converting enzyme
TAK1	Transforming-growth-factor-β-activated kinase 1
TBS	Tris buffered saline
TGFβ	Transforming growth factor β
TIMPs	Tissue inhibitors of matrix metalloproteinases
TIR	Toll-IL-1-receptor
TMB	Tetramethylbenzidine
TNFα	Tumour Necrosis Factor alpha
TNFRI/II	TNF receptor I/II
TRAF6	TNF receptor associated factor
TrkA	Transmembrane kinase p140

Trypsin-EDTA	Trypsin/ethylenediamine tetraacetic acid
TRPV1	Transient receptor potential vanilloid
VEGF	Vascular endothelial growth factor
VSCC	Voltage sensitive Ca^{2+} channels
18S	Eukaryotic 18S rRNA
2-AG	2-arachidonoylglycerol
3D	Three dimensional

Publications and Abstracts

Journal Publications

Dunn SL, Wilkinson JM, Crawford A, Le Maitre CL, Bunning RAD. (2013). Cannabinoid WIN-55,212-2 Mesylate Inhibits Interleukin-1beta Induced Matrix Metalloproteinase and Tissue Inhibitor of Matrix Metalloproteinase Expression in Human Chondrocytes. *Osteoarthritis and Cartilage.* In press.

Wang H, Tian Y, Wang J, Phillips KLE, Binch ALA, **Dunn SL**, Cross A, Chiverton N, Zheng Z, Shapiro IM, Le Maitre CL, Risbud MV. (2013) Inflammatory Cytokines Induce Notch Signaling in Nucleus Pulposus Cells Implications in Intervertebral Disc Degeneration. *Journal of Biological Chemistry.* **288(23)**:16761-74.

Dunn SL, Wilkinson JM, Crawford A, Le Maitre CL, Bunning RAD. (2012) Cannabinoids: Novel Therapies for Arthritis? *Future Medicinal Chemistry.* **4(6):**713-725

Published Abstracts

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Dunn SL, Wilkinson JM, Crawford A, Le Maitre CL, Bunning RAD (2013) Cannabinoid Receptor Expression in Human Osteoarthritic Cartilage. *Proceedings of the British Pharmacological Society*. (Abstract)

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Oral Presentations

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Poster Presentations

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Dunn SL, Wilkinson JM, Crawford A, Le Maitre CL, Bunning RAD (2011). The Effects of Cannabinoids on Chondrocyte Metabolism. *British Society of Matrix Biology*. University of Newcastle.

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1.1 The Human Knee

In the musculoskeletal system the human knee is a biomechanical organ that transits loads between bones, enables movement and supports the body weight. The femur and the tibia, in addition to smaller bones, including the fibula and the patella, compose the bones of the knee joint (Figure 1.1). The knee is composed of two articulations the tibiofemoral and the patellofemoral which are covered by articular cartilage allowing smooth movement of the joint (Flandry and Hommel 2011). The anterior cruciate ligament and the posterior cruciate ligament are found inside the knee joint where they connect the bones of the knee and cross thus stabilising the structure (Amis *et al,* 2006). The meniscus is a crescent shaped fibrocartilage that increases the stability of the femoral tibial articulation in addition to distributing the axial load, absorbing shock and providing lubrication to allow smooth movement of the knee joint (Fox *et al,* 2012). The synovial membrane encapsulates the knee joint and is found between the joint capsule and the joint cavity.

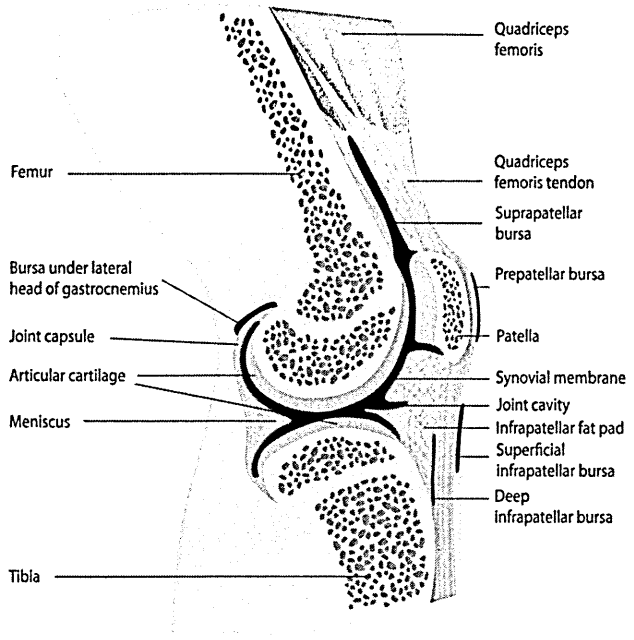

Figure 1.1 The structure of the human knee. The femur and the tibia, in addition to smaller bones, including the fibula compose the knee joint. The medial and lateral condyles are found on the lower extremity of the femur and are covered by articular cartilage. The meniscus is a crescent shaped fibrocartilage that acts to increases the stability of the femoral tibial articulation, distribute the axial load, absorb shock and provide lubrication. The patella intersects with the femur and protects the anterior articulating surface of the knee joint and the cruciate ligaments act to stabilise the joint.

1.2 Structure and Function of the Articular Cartilage

Articular cartilage covers the ends of bones within the joints. The function of the articular cartilage is to provide smooth movement of the articulating joints, allowing the transmission of loads with a low frictional coefficient (Buckwalter and Mankin 1998). The strength of the articular cartilage is dependent on the extensive cross-linking of the collagen fibres in addition to the organisation of the fibrillar architecture within the different zones of the tissue (Eyre 2002). Cartilage is structured into four distinct zones consisting of the superficial tangential zone, the middle transitional zone, the deep radial zone and the calcified zone, with each zone differing in its collagen fibril organisation (Figure 1.2) (Eyre 2002). The superficial zone is composed of collagen fibrils, which are arranged in a tangential array, parallel to the plane of the articulating surface of the cartilage. The middle zone is composed of radial collagen bundles and the deep zone contains, thick radial collagen bundles (Goldring and Marcu 2009). The tidemark is situated in-between the calcified cartilage zone and the subchondral bone acting as a mechanical barrier between the two (Goldring and Marcu 2009).

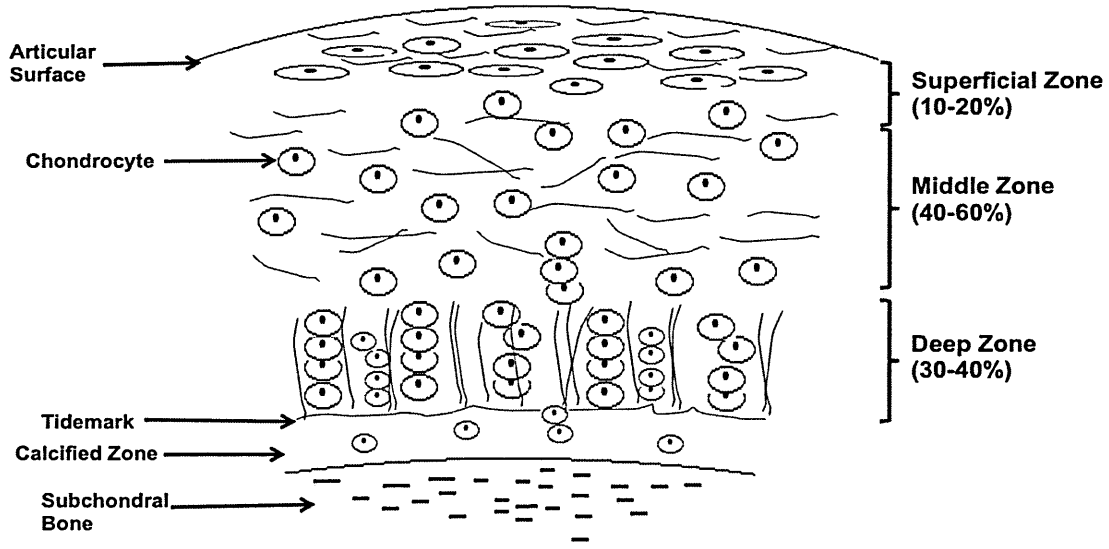

Figure 1.2. Collagen fibre architecture and chondrocyte organisation in articular cartilage zones. The superficial zone is composed of collagen fibrils arranged in a tangential array, parallel to the plane of the articulating surface of the cartilage and chondrocytes are of a flattened morphology. The middle zone is composed of radial collagen bundles and chondrocytes appear rounded. The deep zone is composed of thick radial collagen fibres and the chondrocytes are present in a column formation. The tidemark is situated between the calcified zone and the subchondral bone.

1.2.1 Cartilage Extracellular Matrix

Articular cartilage is composed of a rich extensive collagen matrix, particularly type II, but also types VI, IX and XI (Poole *et al,* 2001). The collagen network is held under high tension by proteoglycans, principally aggrecan (Poole *et al,* 2001). The large aggregating proteoglycan, aggrecan, is retained by the matrix of collagen fibres. This specialized extracellular matrix (ECM) is maintained and synthesised by chondrocytes, the highly differentiated cellular component of the cartilage (Goldring and Marcu 2009). The ECM provides compressive resistance, tensile strength and allows mechanical loading of the joint (Sun 2010). Together the collagen and proteoglycan network maintain articulating cartilage integrity.

1.2.1.1 Collagens

Collagen type II has a half-life of over 100 years and is the most abundant ECM protein in articular cartilage (Verzijl *et al,* 2000). The cross-linked network of collagen and the fibrillar organisation provides cartilage tissue with its tensile strength (Eyre 2002). Collagen fibres are composed of three alpha helices in the conformation of left-handed polyproline II type (PPII) helices. The PPII helical conformation coils in a right-handed formation, with a one-residue stagger to form the triple collagen helix (Eyre 2002). The collagen network within the cartilage changes throughout and the type of collagen and the structural arrangement of collagen fibrils is dependent on the zone and the proximity to the chondrocytes. The ECM network in the interterritorial zone is comprised of mainly type II collagens with other collagens including XI and IX within and on the surface respectively (Goldring and Marcu 2009). The pericellular matrix surrounding the chondrocytes contains primarily type VI collagen microfibrils and allows for the interaction of chondrocytes with the macromolecules of the ECM (Buckwalter and Mankin 1998; Goldring and Marcu 2009).

1.2.1.2 Proteoglycans

Proteoglycans of which aggrecan is the major proteoglycan of cartilage, protect the collagen network. The half-life for aggrecan core protein is between 3 and 24 years (Maroudas *et al,* 1998). Aggrecan's core protein is composed of three globular domains, G1, G2 and G3 (Figure 1.3). The large extended region between G2 and G3 allows for the binding of glycosaminoglycans (GAGs) (Kiani *et al,* 2002). Aggrecan monomers are attached to hyaluronic acid (HA) polymers via the G1 domain and link protein. Aggrecan provides articular cartilage with its

osmotic properties because of its high polyanionic charge allowing the retention of water thus providing the articular cartilage with the ability to resist compressive loads (Roughley and Lee 1994). Smaller proteoglycans including biglycan, decorin and fibromodulin bind to other matrix macromolecules to stabilise the ECM (Buckwalter and Mankin 1998). The ECM is also composed of other non-collagenous molecules including, matrillins, cartilage oligomeric matrix protein (COMP) and elastin (Goldring and Marcu 2009).

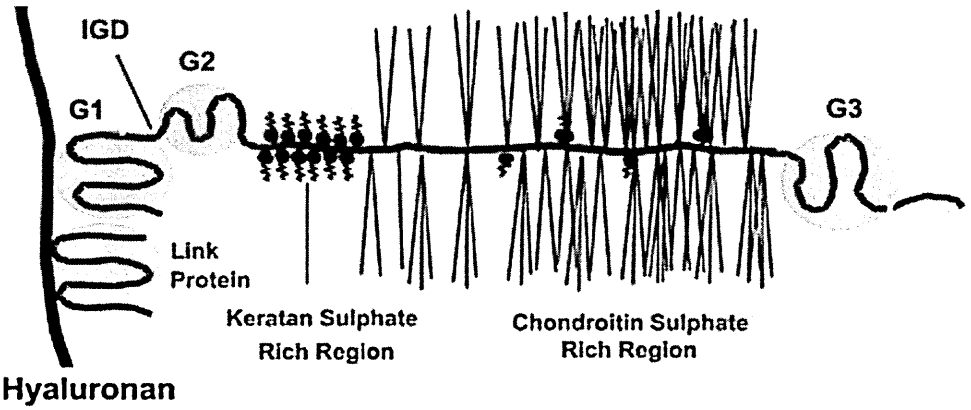

Figure 1.3 The structure of aggrecan linked to hyaluronic acid and stabilised by link protein. Modified from Porter *et al,* (2005).

1.2.2 Chondrocytes

Chondrocytes are the only cellular component of adult articular cartilage and are fully differentiated cells that remain after the formation of the articular cartilage matrix. In normal cartilage the chondrocytes are in a quiescent state with little turnover of the ECM (Loeser *et al,* 2012). These specialised cells reside in an avascular environment within the joint and depend on the diffusion of nutrients through the ECM to survive in their harsh environment (Goldring 2000). Their role is to maintain the balance between the rate of synthesis of ECM and its degradation and subsequent loss into the synovial fluid (Goldring 2000).

The chondrocyte's role, morphology and organisation differ in each zone of the cartilage (Figure 1.2). These changes may be attributed to different mechanical influences. The chondrocytes that reside in the superficial zone are of a flattened morphology, the middle zone chondrocytes are rounded and the deep zone chondrocytes are present in stacked groups (Goldring and Marcu 2009). In

addition to having different organisation within the cartilage, chondrocytes have also been shown to have different gene expression profiles (Fukui *et al,* 2008). Chondrocytes that reside in the superficial zone of the cartilage produce lubricin a glycoprotein which crossed-links with HA to provide lubrication, allowing smooth movement of the joint at the articular surface, in addition to providing protection from mechanical forces and loads (Greene *et al,* 2011).

1.3 Extracellular Matrix Turnover in Articular Cartilage

Factors that maintain ECM turnover in articular cartilage are poorly understood, since the chondrocytes do not divide rapidly and the ECM isolates them from each other (Goldring and Marcu 2009). However, ECM turnover in cartilage is thought to be maintained by chondrocytes via the production of matrix degrading enzymes, the matrix metalloproteinases (MMPs) and the 'a disintegrin and metalloproteinase with thrombospondin motifs' (ADAMTSs) which cleave collagens and proteoglycans which are subsequently released into the synovial fluid (Murphy and Nagase 2008). The activity of matrix degrading enzymes is tightly regulated by tissue inhibitors of matrix metalloproteinases (TIMPs). During normal cartilage ECM matrix turnover there is thought to be a balance in the levels of MMPs and TIMPs (Burrage *et al,* 2006a; Martel-Pelletier *et al,* 1994b). Under normal homeostatic conditions proteases combine to form complex regulatory networks in order to maintain ECM turnover and under normal physiological conditions MMPs are involved in cartilage ECM turnover, remodelling and repair (Goldring and Marcu 2009).

1.3.1 Matrix Metalloproteinases

MMPs are family of 23 active zinc-dependent proteinases that at neutral pH specifically degrade triple helical collagens and proteoglycans (Burrage *et al,* 2006a; Murphy *et al,* 2002; Cawston and Young 2010). MMPs share a common domain structure and have a catalytic domain, a hinge region, a signal peptide, a propeptide and a C-terminal domain (Clark *et al,* 2008). MMPs are secreted from the chondrocyte in latent form as pro-MMPs requiring activation extracellularly (Murphy *et al,* 2002). Zinc is present in the catalytic domain and pro-MMPs are maintained in a latent form by interaction of a conserved cysteine residue in the pro-domain with the catalytic zinc in the active site (Cawston and Young 2010). All MMPs except MMP-7, -23 and -26 have a hinge region which links to a haemopexin-like C-terminal domain that is involved in the substrate and inhibitor

binding specificity (Clark *et al,* 2008). In addition to cleaving ECM molecules, MMPs also cleave other proteinases. MMP-3 is able to activate MMP-1 via cleavage of the pro-domain (Vincenti and Brinckerhoff 2002). MMPs are divided into groups depending on the ECM substrates they cleave and include the stromelysins, collagenases, gelatinases and membrane type MMPs (Clark *et al,* 2008).

1.3.2 ADAMTSs

The ADAMTS family is composed of 19 members, and are secreted metalloproteinases containing a signal peptide, a prodomain, a thrombospondin type I motif, a cysteine rich domain, a spacer domain and a second thrombospondin motif (Porter *et al,* 2005). ADAMTS-1, -4, -5, -8 ,-9, -15, -16 and -18 all have the ability to cleave aggrecan, in addition to other proteoglycans including versican and brevican of which ADAMTS-4 and -5 are most active in the degradation of aggrecan (Murphy and Nagase 2008;Gendron *et al,* 2007).

1.3.3 Tissue Inhibitors of Matrix Metalloproteinases

TIMPs are a family of 21-30 kDa proteinase inhibitors that are produced by chondrocytes and fibroblasts and regulate the activity of MMPs (Burrage *et al,* 2006). The TIMP family of inhibitors consists of 4 members including TIMP-1, -2, -3 and -4. Between the four isoforms TIMPs have high levels of sequence divergence, however all TIMPs bind to and inhibit MMPs (Brew *et al,* 2000). TIMP inhibition of MMPs requires the non-covalent binding to the MMPs active site with a 1:1 stoichiometry and interactions between MMPs and TIMPs are irreversible under physiological conditions (Burrage *et al,* 2006). In addition to inhibiting MMPs, TIMP-1 also inhibits ADAMTS-10 and TIMP-3 inhibits ADAMTS-1, -4, -5 -10, -12 and -17 (Baker *et al,* 2002).

1.3.4 Chondrogenesis

During skeletal development chondrogenesis is the process via which cartilage is developed. Chondrogenesis involves the biological process of endochondral ossification, which leads to the formation of the skeleton (Goldring *et al,* 2006). The early limb bud forms as mesoblast, undifferentiated mesenchymal cells migrate to the limb bud and condense to form the cartilage anlage (Goldring 2012). Condensation of mesenchymal cells expressing collagens I, III and V and chondroprogenitor cell differentiation with cells expressing cartilage specific genes including collagens II, IX and XI results in chondrogenesis (Goldring 2012).

Mesenchymal condensation is followed by chondrocyte differentiation, chondrocyte proliferation, development of the cartilage template and chondrocyte hypertrophy (Zuscik *et al,* 2008). During chondrocyte hypertrophy the cells enlarge and terminally differentiate, mineralise and finally undergo apoptosis thus permitting endochondral ossification, a process that involves the formation of bone via resorption of the calcified hypertrophic cartilage (Zuscik *et al,* 2008). Replacement of cartilage with bone requires angiogenesis mediated by vascular endothelial growth factor (VEGF) (Goldring *et al,* 2006). A similar process also occurs in the postnatal growth plate and is termed secondary centre of ossification as opposed to primary ossification, which occurs during fetal development in the central part of the developing bone (Zuscik *et al,* 2008). The secondary centre for ossification separates the articular cartilage and the mature growth plate cartilage.

Chondrogenesis is under tight regulation and involves cellular interactions with the surrounding matrix, growth and differentiation factors, the initiation and suppression of cellular signalling pathways and the recruitment of transcription factors to control and orchestrate the expression of specific genes in a temporal and spatial pattern (Goldring *et al,* 2006). The transcription factors Sox-9 and Runx2 play a role in the formation of mesenchymal condensation, maintaining the chondrocyte phenotype, control of chondrogenic differentiation and regulating the expression of ECM genes particularly cartilage specific collagen type II (Lefebvre *et al,* 1997; Akiyama *et al,* 2002). Furthermore, Sox-9 and Runx2 determine the fate of the chondrocytes to remain within the articulating cartilage or undergo ossification respectively (Goldring and Marcu 2009).

1.3.5 Vasculature

Articular cartilage is an avascular tissue with low availability of oxygen and glucose. Since the chondrocytes reside in an avascular and aneural environment they rely on the diffusion of nutrients from the surrounding tissue and express glucose transporter proteins GLUT3 and GLUT7 to facilitate in the transport of glucose (Mobasheri *et al,* 2005). Chondrocytes reside at a low oxygen tension with less than 1% oxygen present in the deep zones of the articular cartilage and chondrocytes adapt to their low oxygen environment via the upregulation of hypoxia-inducible factor-1-alpha (HIF-1α), a transcription factor which is known to induce the expression of GLUTs and angiogenic factors including VEGF (Pfander

and Gelse 2007). In addition, in normal articular chondrocytes DNA damage 45 beta (GADD45β) acts as a survival factor in addition to regulating chondrocyte homeostasis and promoting collagen expression (Ijiri *et al,* 2008). Therefore, chondrocytes residing in an avascular environment survive by modulating the intracellular expression of survival factors to maintain the ECM (Goldring and Marcu 2009).

1.3.6 Cytokines

Cytokines are a large group of peptides with a diverse range of biological actions and are produced by a variety of cell types in response to different stimuli. Cytokines can be proinflammatory, anti-inflammatory and involved in the proliferation, differentation and activation of immune cells and hematopoiesis (Dinarello 2007).

1.3.6.1 Interleukin-1

Interleukins are a family of secreted proteins, which bind to specific receptors, expressed on numerous cell types (Akdis *et al,* 2011). Interleukin-1 beta (IL-1β) is secreted in pro-form (pro-IL-1β) as a biologically inactive precursor requiring post-translational cleavage by the intracellular IL-1β-converting enzyme (caspase-1), generating the mature active cytokine (Mosley *et al* 1985). IL-1β induces its biological effects via binding to the IL-1 cell surface receptor IL-1 receptor type I (IL-1R1). In addition a second agonist IL-1α also has activity at IL-1R1. Similarly, IL-1α is secreted in pro-form as a precursor however in contrast to IL-1β pro-IL-1α is biologically active (Mosley *et al* 1985). Following IL-1α/β binding specifically to the extracellular ligand binding chain, a second accessory chain is recruited and IL-1R1 forms a heterodimeric complex with IL-1 receptor accessory protein (IL-1RAcP) to allow signal transduction (Figure 1.4) (Boraschi and Tagliabue 2013). The ligand binding chain and accessory chains are composed of a cytosolic Toll-IL-1-receptor (TIR) domain (Boraschi and Tagliabue 2013). IL-1β also binds to a second cell surface receptor IL-1RII which is a decoy receptor as it is unable to transduce a signal. An endogenous inhibitor of IL-1β induced responses is IL-1 receptor antagonist (IL-1Ra), which binds to both IL-1R1 and IL-1RII without inducing a signal (Arend *et al,* 1998).

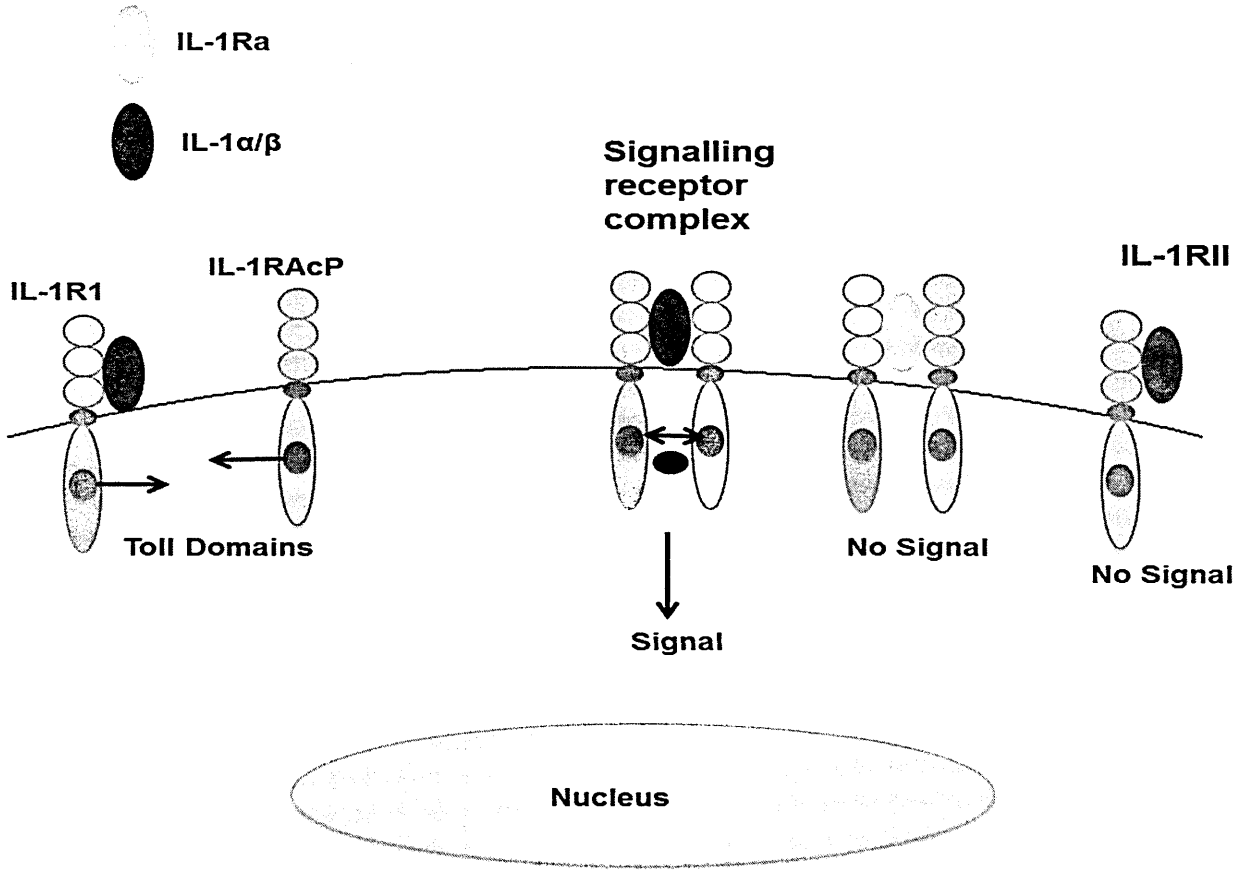

Figure 1.4 IL-1 Receptor Signalling. IL-1α/β binds to the cell membrane bound receptor IL-1 receptor 1 (IL-1R1), leading to the recruitment of IL-1 receptor accessory protein (IL-1AcP) forming a heterodimer complex inducing an intracellular signal. IL-1Ra bound receptors are unable to form the heterodimeric complex therefore no signal is produced. IL-1α/β binds to a second cell surface receptor IL-1RII which is referred to as a decoy receptor as it is unable to transduce a signal.

1.3.7 Tumour Necrosis Factor α

Tumour Necrosis Factor alpha (TNFα) is pro-inflammatory cytokine and is produced by many cell types, in response to a broad range of stimuli. TNFα is synthesised as a biologically active protein and is displayed on the extracellular membrane and exists as a soluble form, proteolytically shed from the plasma membrane via cleavage by the TNFα converting enzyme (TACE/ADAM-17) a member of the A Disintegrin And Metalloprotease enzymes (Black 2002). TNFα induces its effects via binding to cell membrane surface receptors including, TNF receptor I (TNFRI or p55) and TNF receptor II (TNFRII or p75) (Idriss and Naismith 2000). Both receptors exist in cell-associated and soluble forms also shed by TACE/ADAM-17. Furthermore, both receptors are shed by MMPs and receptors are involved in signal transduction, however TNFRI is thought to be primarily involved in TNFα induced activity in joint cells (Kapoor *et al,* 2011).

1.4 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is predominantly an inflammatory joint disease, characterised by inflammation of the synovium which lines the joint cavity with infiltration of inflammatory cells into the synovial fluid. The enlarged synovium becomes vascularised and may extend over other joint tissues such as cartilage and bone, forming a pannus that produces degradative enzymes and inflammatory cytokines, which leads to the breakdown of the ECM of the articular cartilage (Otero and Goldring 2007;Rannou *et al,* 2006).

1.5 Osteoarthritis

Osteoarthritis (OA) is the most common form of arthritis and is a major cause of pain and disability in older adults with around 8 million people in the UK suffering from symptomatic OA (Arthritis Research UK, 2012). OA constitutes a significant economic burden, which is increasing with an ageing population (Arthritis Research UK, 2012). OA is a progressive degenerative disease of the joint and a key pathological feature of OA is loss of articular cartilage, which contributes to the pain and joint deformity experienced by patients (Goldring and Goldring 2007). The aetiology of OA is poorly understood and it is thought to involve multiple factors including, mechanical, inflammatory, genetic and metabolic. Inflammation of the synovium also occurs during OA and contributes

to cartilage breakdown but to a lesser degree than seen in RA. Although OA and RA have different cellular mechanisms, cartilage breakdown is a key feature of both diseases. OA affects multiple joints including hands, knees, the spine and hips (Goldring and Goldring 2007). To date treatments for OA mainly address the symptoms, therefore, it is important to develop more effective disease modifying agents in order to prevent disease progression (Goldring 2006).

1.6 Pathogenic Factors in OA

Cartilage degradation is the main pathological feature of arthritis however it is now evident that other joint tissues including the subchondral and trabecular bone, the synovium and calcified bone are affected and involved in the pathogenesis (Goldring and Goldring 2010). OA is often considered as a degenerative joint disease; however other processes including mechanical, biochemical factors and the remodelling of joint tissue due to inflammation are involved. Factors that may lead to the onset of OA include; obesity, sex, age, genetics, abnormal loading of the joint and previous injury (Zhang and Jordan 2010).

Multiple changes in the pathological features of joint structures can be seen in OA. These changes encompass the whole of the joint and include degradation of the cartilage, thickening of the subchondral bone, osteophyte formation, and the formation of pannus (Loeser *et al,* 2012). OA is now seen as a multifactorial disease involving multiple tissues, with changes also observed in nerves, periarticular muscle, bursa and fat pads (Loeser *et al,* 2012).

1.6.1 Chondrocyte Metabolism in OA

During OA the chondrocytes become activated in response to mechanical stress, abnormal loading of the joint and inflammatory cytokines and there is a shift in the equilibrium between anabolic and catabolic activities which maintains the ECM in healthy cartilage (Goldring and Marcu 2009; Patwari *et al,* 2003; Goldring and Berenbaum 2004). Factors that contribute to changes in chondrocyte metabolism during OA include: increased stress during aging, abnormal mechanical loading of the joint, changes in the surrounding ECM and the up regulation of proinflammatory mediators (Goldring and Goldring 2010). Primary human chondrocytes derived from OA cartilage and non-arthritic aged

cartilage proliferate at a slower rate compared to chondrocytes derived from young normal cartilage and display senescence (Price *et al,* 2002). The morphology of the chondrocytes is also altered in OA and non-arthritic aged cartilage compared to young normal cartilage derived chondrocytes (Dozin *et al,* 2002). During OA chondrocytes proliferate, form clusters and increase their production matrix degrading enzymes. This switch from normal quiescent resting chondrocytes may be attributed to an injury response, which involves a recapitulation of processes that are usually only activate during fetal development, leading to the remodelling of the ECM, hypertrophy and cartilage calcification (Figure 1.5) (Aigner *et al,* 2007).

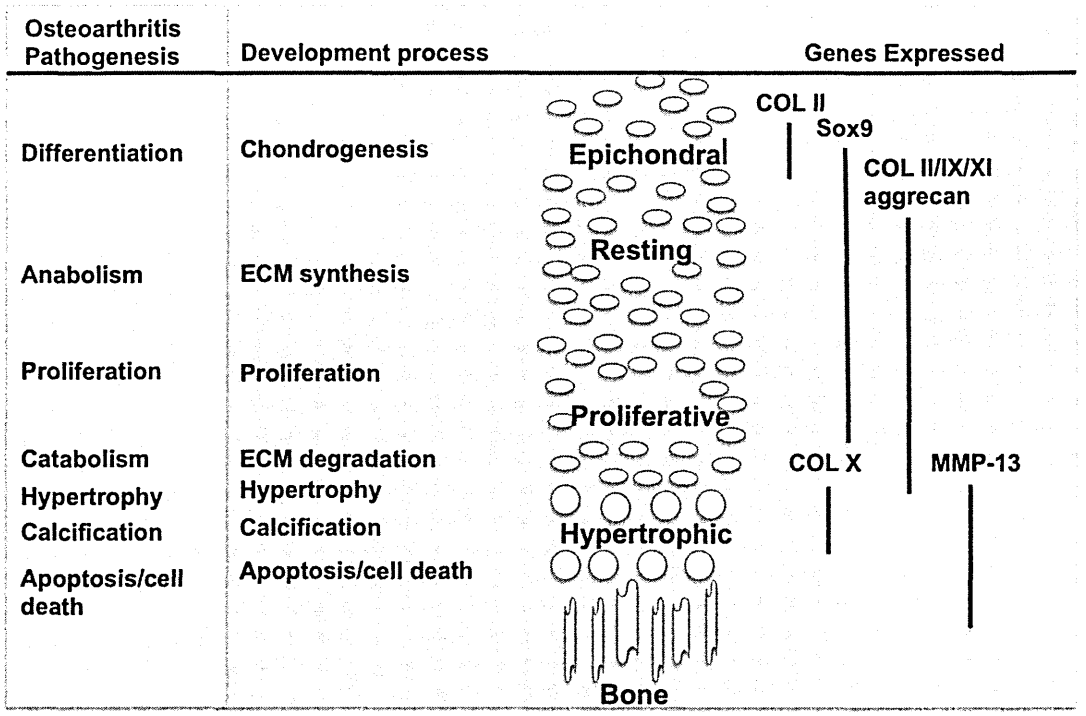

Figure 1.5 Chondrocyte behaviour during the development process and osteoarthritis. Processes that occur during fetal chondrogenesis are also thought to occur during osteoarthritis such as changes in chondrocyte differentiation, anabolism, proliferation, catabolism, hypertrophy, cartilage calcification and apoptosis. These changes are identified by changes in genes that are also related to events during fetal development including sox-9 indicating chondrocyte differentiation, collagen type II a cell marker for chondroprogenitors, collagen type X a marker for hypertrophic chondrocytes and MMP-13 also linked to hypertrophy in addition to matrix breakdown. Although these processes are similar, chondrogenesis is a tightly controlled and structured development process, whilst osteoarthritis is an uncoordinated degenerative process. (Modified from Aigner *et al* (2007)).

1.6.2 Anabolic Metabolism

A decrease in ECM production particularly collagen type II and aggrecan during OA induces changes in the biomechanical properties of the tissue. IL-1β is known to affect anabolic metabolism in OA, reducing the expression of collagen type II and aggrecan (Goldring *et al,*1994; Chadjichristos *et al,* 2003; Stove *et al,* 2000). Interestingly, during early OA, chondrocytes are known to increase their synthesis of cartilage ECM components including collagens type II, IX and X, aggrecan and pericellular collagen type VI, a process is thought to be an attempt to regenerate the cartilage matrix (Goldring and Goldring 2007). This process is thought to involve similar molecular mechanisms to those occurring during fetal development (Aigner *et al,* 2007) (Figure 1.5). However, unlike chondrogenesis, which is a highly regulated process, similar pathways activated during OA are uncoordinated degenerative processes.

1.6.3 Catabolic Metabolism

During OA there is an increase in catabolic factors produced by the chondrocytes in addition to cells of the synovium compared to normal. During disease progression there is inappropriate elevation in the expression of matrix degrading enzymes predominantly MMP-1, -3 and -13 without an increase in their inhibitors TIMPs, which in part is thought to contribute to cartilage degradation (Martel-Pelletier *et al,* 1994). MMPs are involved in tissue remodelling, repair and the formation of the surrounding ECM under normal conditions (Burrage *et al,* 2006). However a shift in equilibrium between anabolic and catabolic activities occurs in OA, favouring the catabolic processes, resulting in net cartilage breakdown.

1.6.3.1 Cytokines and Chemokines in OA

During OA proinflammatory cytokines are predominantly involved in the disruption of metabolism and the upregulation of catabolic factors. IL-1β and TNFα are thought to be the main cytokines involved in the pathogenesis of OA, however other cytokines including IL-6, IL-15, IL-17, IL-18 and IL-21 and chemokines IL-8, CCL5 (RANTES), CCL2 and CXCL 1 are known to contribute to the disease progression (Table 1.1) (Kapoor *et al,* 2011).

Cytokine	Role in OA	Reference
IL-1β	• Increased in OA cartilage, subchondral bone and the synovium. • Suppresses the synthesis of ECM molecules including collagen type II and aggrecan by chondrocytes. • Induces the release of MMP-1, -3, -9 and -13 and other pro-inflammatory cytokines including IL-6 and chemokines IL-8, CCL5 and CCL2	Goldring *et al* 1994; Chadjichristos *et al,* 2003 Stove *et al,* 2000; Lefebvre *et al,* 1990; Reboul *et al,* 1996; Guerne *et al,* 1990; Lotz *et al,* 1992
TNFα	• Increased in OA cartilage, subchondral bone and the synovium. • Supresses the synthesis of ECM molecules including collagen type II and aggrecan by chondrocytes. • Induces the release of MMP-1, -3,-9 and -13 and other pro-inflammatory cytokines including IL-6 and chemokine IL-8	Saklatvala 1986; Lefebvre *et al* 1990; Rebou *et al* 1996; Guerne *et al* 1990; Lotz *et al* 1992.
IL-6	• Increased in OA synovial fluid • Induces the expression of MMP-1 and -13 synergistically with IL-1β and onocostin M. • Reduces collagen type II expression	Kaneko *et al,* 2000; Cawston *et al,* 1998 Rowan *et al,* 2001; Poree *et al,* 2008
IL-15	• Increased in early OA synovial fluid and is correlated with MMP-1 and -3 levels	Scanzello *et al,* 2009
IL-17	• Induces IL-1β, TNFα, IL-6, NO and MMP-13 in chondrocytes • Inhibits chondrocyte proteoglycan production	Martel-Pelletier *et al,* 1999; Lubberts *et al* 2000
IL-18	• Increased in human OA chondrocytes • Induces NO, COX-2, IL-6 and proteoglycan release	Olee *et al,* 1999
IL-21	• Increased in the synovial fluid of early OA	Scanzello *et al,* 2009
Chemokine IL-8	• Increased in OA synovium and chondrocytes • Induces MMP-13 production • Synergises with TNFα to increase PGE_2 and COX-2	Kaneko *et al,* 2000; Merz *et al,* 2003 Attur *et al,* 1998; Alaaedine *et al,* 1999
Chemokines CCL5, CXCL 1 and CCL2	• Increased in OA • Induces MMP-1, IL-6 and iNOS • Induces proteoglycan breakdown	Alaaeddine *et al,* 2001; Alaaeddine *et al,* 1999

Table 1.1 Cytokines and chemokines involved in the pathogenesis of osteoarthritis

1.6.3.2 Interleukin-1β in OA

Pro-inflammatory cytokine IL-1β plays a major role in the pathogenesis of OA (Kapoor *et al,* 2011). Although cells of the joint residing in normal tissue produce low levels of IL-1β, increased levels of IL-1β have been identified in the cartilage, synovial fluid, synovial membrane and subchondral bone in patients with OA (Fernandes *et al,* 2002). Interestingly elevated levels of IL-1β were found in the superficial zone of articular cartilage (Fan *et al,* 2007), furthermore increased IL-1β levels are associated with an increase in IL-1β-converting enzyme in human OA tissues (Saha *et al,* 1999). Although chondrocytes are known to produce IL-1β, increased levels in the upper zones of the cartilage may also indicate diffusion of IL-1β from the synovial fluid (Fan *et al,* 2007). However it is evident that inflammatory responses during OA are also attributed to the autocrine production of IL-1β by chondrocytes (Attur *et al,* 1998). Human OA chondrocytes secrete sufficient amounts of functionally active IL-1β, to induce pro-inflammatory mediators including nitric oxide (NO), and prostaglandin E_2 (PGE_2) compared to normal chondrocytes (Attur *et al,* 1998). During OA IL-1β stimulates chondrocytes to secrete matrix-degrading enzymes principally MMP-1, -3 and -13, inducing cartilage degradation (Figure 1.6) (Lefebvre *et al,* 1990; Reboul *et al,* 1996; Mengshol *et al,* 2000).

Chondrocytes and synovial cells are known to produce IL-1Ra and it has been shown to protect against inflammatory and catabolic responses (Seitz *et al,* 1994; Palmer *et al,* 2002). In addition, OA joint cells are more responsive to IL-1β stimulation, since IL-1R1 expression is increased in OA chondrocytes and synovial fibroblasts compared to normal non-arthritic cells (Martel-Pelletier *et al,* 1992; Sadouk *et al,* 1995).

In addition to inducing catabolic factors during OA, IL-1β also suppresses anabolic activities of chondrocytes and reduces the expression of collagen II and aggrecan (Goldring *et al,* 1994; Chadjichristos *et al,* 2003; Stove *et al,* 2000). In human OA cartilage explants, IL-1β reduced the synthesis of both TIMP-1 and -2 (Martel-Pelletier *et al,* 1994). Although playing a significant role in cartilage degradation the importance of IL-1β in maintaining cartilage homeostasis has been highlighted, as IL-1β gene deletion accelerated the development of OA lesions in a mouse model (Clements *et al,* 2003).

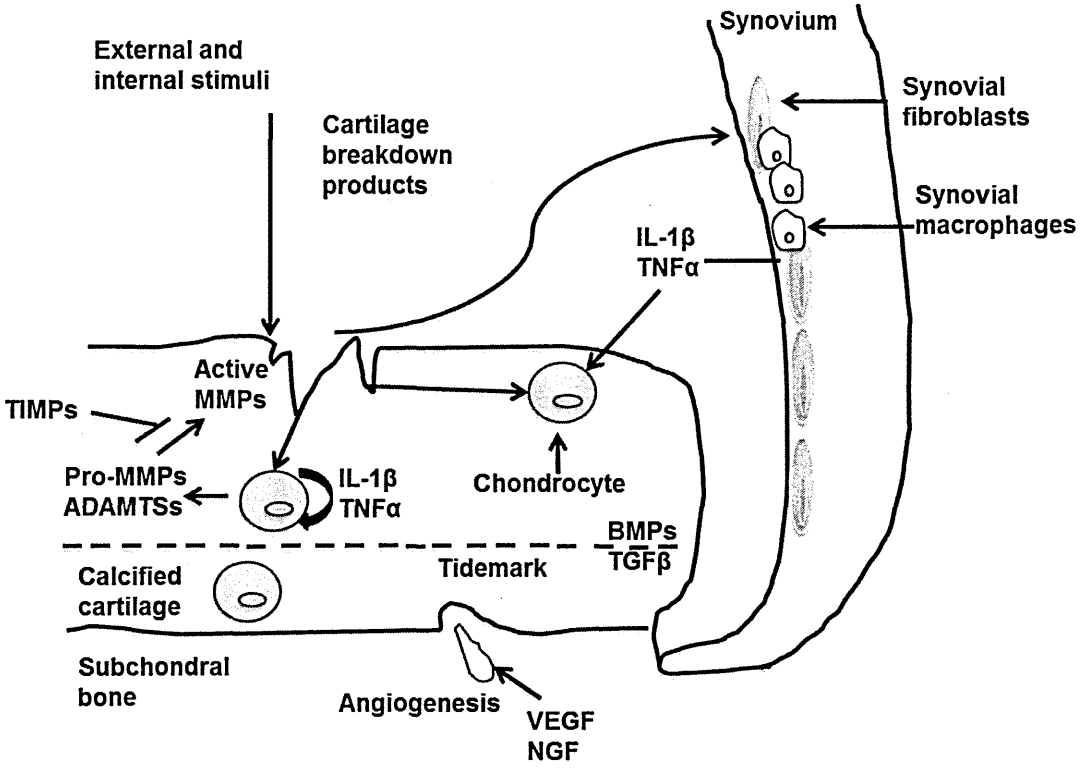

Figure 1.6 Processes of cartilage breakdown in osteoarthritis. External and internal stimuli induce catabolic cytokines including interleukin 1 beta (IL-1β) and tumour necrosis factor alpha (TNFα) which are secreted by cells of the synovium or chondrocytes resulting in the upregulation of matrix degrading enzymes including matrix metalloproteinases (MMPs) and ADAMTSs. Matrix breakdown products can feedback and regulate these cellular events further inducing cartilage breakdown. Anabolic factors including BMPs and TGFβ are increased and lead to the formation of osteophytes. Other changes including increased chondrocyte proliferation and hypertrophy increase cartilage calcification with advancement of the tidemark occurring. Angiogenesis occurs at the osteochondral junctions mediated by vascular endothelial factor (VEGF), in addition to nerve growth factor (NGF) contributing to OA pain.

1.6.3.3 Tumour Necrosis Factor α in OA

During OA TNFα is associated with mediating the inflammatory response (Kapoor *et al,* 2011). TNFα is synthesised by chondrocytes, bone cells and synovial cells and is elevated in the cartilage, synovium membrane, synovial fluid and subchondral bone in patients with OA. Furthermore TNFRI expression is increased in human OA chondrocytes and synovial fibroblasts compared to normal cells (Alaaeddine *et al,* 1997). TNFα induces chondrocytes to produce MMP-1, -3 and -13, thus contributing to cartilage breakdown. Additionally, TNFα inhibits the synthesis of proteoglycan and stimulates resorption in cartilage explants (Saklatvala 1986).

1.6.3.4 Chemokine IL-8 in OA

Chondrocytes express chemokines and chemokine receptors, which may in part contribute to the induction of cartilage breakdown by the induction of catabolic factors (Borzi *et al,* 2000). Chemokine IL-8 is thought to contribute to cartilage breakdown in OA and is elevated in human OA chondrocytes and the synovial fluid of OA patients (Kaneko *et al,* 2000; Attur *et al,* 1998). In human and bovine chondrocytes MMP-13 secretion is up-regulated by IL-8 (Merz *et al,* 2003), suggesting IL-8 plays a role in cartilage breakdown via the upregulation of matrix degrading enzymes. In addition IL-1β has a direct effect on chemokine production; in human articular chondrocytes IL-1β induces the production of chemokine IL-8 that is thought to contribute to cartilage breakdown and inflammation via the recruitment and degranulation of neutrophils (Lotz *et al,* 1992; Elford and Cooper 1991). Furthermore, in human OA synovial fibroblasts IL-8 synergises with TNFα to increase PGE_2 secretion and COX-2 synthesis (Alaaeddine *et al,* 1999). These findings suggest that IL-8 interacts with pro-inflammatory cytokines to induce inflammatory responses in OA.

1.6.3.5 Nitric Oxide

NO is synthesised from L-arginine oxidiation by inducible nitric oxide synthase (iNOS) (Abramson *et al,* 2001). An increase in IL-1β and TNFα during OA stimulates the up-regulation of proinflammatory NO via iNOS, resulting in damage to surrounding cells and tissue and induction of apoptosis (Abramson *et al,* 2001). NO is thought to be involved in mediating IL-1β induced responses including the upregulation of MMPs and inhibition of collagen and aggrecan expression (Abramson 2008). Therapies, which target inhibition of iNOS, have

been shown to reduce joint damage and inflammation in animal models of adjuvant-induced arthritis (AIA) and collagen-induced arthritis (Pelletier *et al,* 1999).

1.6.3.6 Prostaglandin E_2

Cyclooxygenases (COX) enzymes of which there are two isoforms COX-1 and -2, catalyse the conversion of arachidonic acid to prostaglandins including the pro-inflammatory mediator PGE_2 (Martel-Pelletier *et al,* 2003). Prostaglandins are elevated in the synovial fluid and cartilage from patients with OA (Martel-Pelletier *et al,* 2003; Hardy *et al,* 2002). During OA IL-1β and TNFα increase the synthesis of PGE_2 by chondrocytes via the upregulation of COX-2 (Martel-Pelletier *et al,* 2003). Furthermore, in human cartilage explants PGE_2 inhibited proteoglycan synthesis and induced cartilage degradation, in addition, PGE_2 augmented IL-1β induced MMP-13 expression (Attur *et al,* 2008), suggesting that PGE_2 contributes to cartilage breakdown during OA.

1.6.4 IL-1β Signalling in OA

The pro-inflammatory and catabolic actions of IL-1β are mediated by the activation of mitogen activated protein kinases (MAPKs) including, Jun N-terminal kinase (JNK), the extracellular signal-regulated kinases (ERKs) and the p38 kinases and nuclear factor κB (NFκB) signalling pathways during OA (Goldring *et al,* 2008). Activation of these signalling pathways leads to the up-regulation of catabolic genes, including genes for MMP-1, -3 and -13 and inflammatory genes including iNOS and COX-2 (Kapoor *et al,* 2011; Burrage *et al,* 2006).

1.6.4.1 Mitogen Activated Protein Kinases

The MAPKs are a family of serine/threonine protein kinases and during OA they mediate IL-1β induced intracellular signalling pathways. In MAPK mediated signalling pathways JNK and p38 are primarily phosphorylated in response to external stimuli including proinflammatory cytokines, apoptotic signals and osmotic stress and the ERKs in reponse to proinflammatory cytokines and growth factors (Davis 2000; Garrington and Johnson 1999; Vincenti and Brinckerhoff 2002). Following IL-1β stimulation the MAPK kinase kinases (MAPKKK, MEKK or MKKK) are activated and in turn phosphorylate other MAPK kinases (MAPKK, MEK or MKK) that subsequently phosphorylate and

activate MAPKs including JNK, ERK1/ERK2 and p38 (Figure 1.7) (Vincenti and Brinckerhoff 2002). MAPKs translocate to the nucleus activating a number of transcription factors involved in the pathogenesis of OA (Figure 1.7).

MMP synthesis is tightly regulated at the gene level leading to cell and tissue specific expression. During OA MMP-1, -3, -9 and -13 genes are induced by IL-1β and TNFα. MMP-1, -3, -7, -9, -10, -12, -13, -19 and -26 are regulated by similar mechanisms at the transcription level and their promoters contain an activator protein 1 (AP-1) binding site which is -73 bp up stream of the transcriptional start site and a TATA box located at -30 bp (Benbow and Brinckerhoff 1997; Yan and Boyd 2007). JNKs and ERKs phosphorylate c-Jun, which in turn forms c-fos/c-Jun heterodimers, or c-Jun/c-Jun homodimers that together compose the AP-1 protein complex (Figure 1.7) (Burrage *et al,* 2006). AP-1 proteins cooperate with other transcription factors including erythroblastosis twenty-six (Ets) proteins, which are also activated by ERK to induce the transcription of MMPs (Vincenti and Brinckerhoff 2002; Janknecht *et al,* 1995). Furthermore, p38 phosphorylation induces the c-Jun promoter via the activation of activating transcription factor 2 and the ternary complex factor Elk-1 leading to the activation of c-fos promoter (Davis 2000).

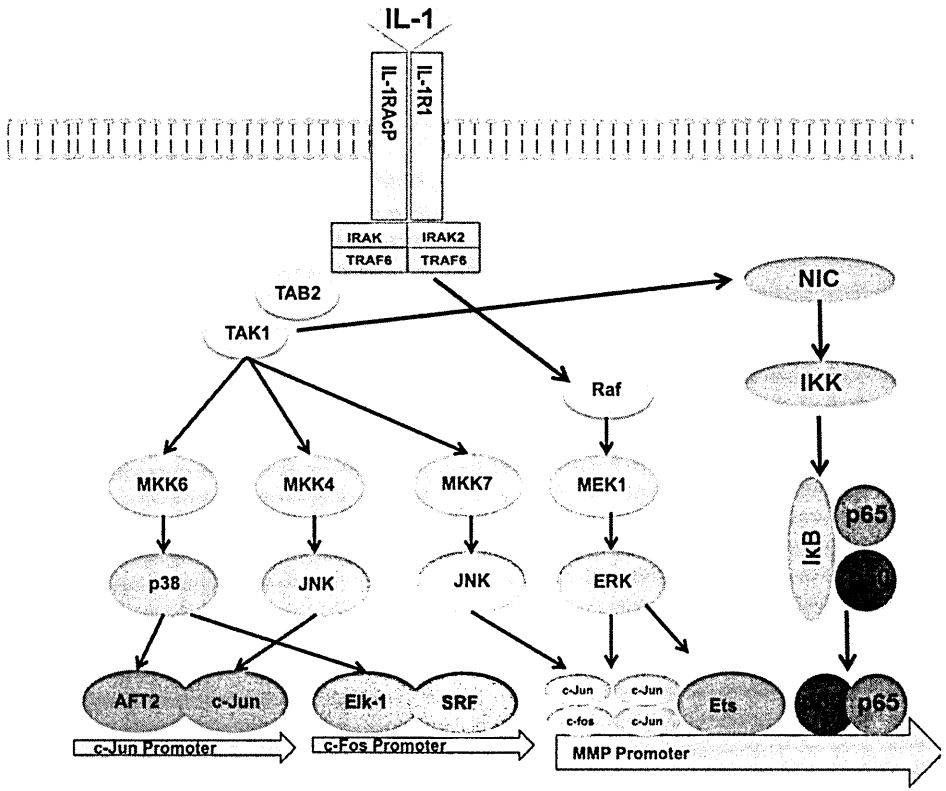

Figure 1.7 IL-1α/β signalling pathways in chondrocytes. IL-1α/β regulates the transcription of MMPs through signal transduction pathways including mitogen-activated protein kinases (MAPKs) and nuclear factor kappa B (NFκB). IL-1α/β activates MAPK kinase kinases (MAPKKKs), transforming-growth-factor-β-activated kinase 1 (TAK1) and Raf which phosphorylate MAPK kinases (MKKs:MKK6, MKK4, MKK7 and MEK) which in turn phosphorylate and activate MAPKs including, Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK) which translocate to the nucleus. MAPKs phosphorylate and activate transcription factors that contribute to the expression of MMPs and other genes including c-fos and c-Jun. The NFκB signalling pathway involves the activation of TAK1 following IL-1α/β stimulation which in turn phosphorylates and activates NFκB-inducing kinase (NIK). NIK activates inhibitor of κB kinase which phosphorylates inhibtor of κB (IκB) targeting it for ubiquitination and subsequent proteasome degradation, thus, allowing the translocation of NFκB to the nucleus, where it binds to κB consensus sequences inducing the expression of MMPs (Modified from Vincenti and Brinckerhoff (2002)).

1.6.4.2 Nuclear Factor κB

NFκB is a transcription factor and its activation regulates the expression of cytokines and chemokines, inflammatory mediators and matrix degrading enzymes associated with the pathogenesis of OA (Marcu *et al,* 2010). IL-1β activity is also regulated by NFκB and is required for chondrocytes to initiate the expression of genes involved in OA pathogenesis (Goldring and Otero 2011). The activity of NFκB is tightly regulated and is dependent on its cellular localisation. NFκB mediated transcription control is via the assembly of homodimers and heterodimers of 5 different NFκB proteins including RelA/p65, RelB, cRel, NFκB/p105, and NFκB/p100 (Marcu *et al,* 2010). NFκB is held in the cytoplasm of unstimulated cells via direct interaction with different inhibitory kappa B (IκB) proteins of which IκBα and IκBβ predominantly bind to NFκB containing p65 (Valovka and Hottiger 2011). Following stimulation from pro-inflammatory or stress-like responses, IκB kinase (IKK) phosphorylates the site specific amino-terminal of IκB, targeting it for ubiquitination and subsequent proteasome degradation, thus, allowing the translocation of NFκB to the nucleus, where it binds to κB consensus sequences inducing the expression of target genes including MMPs (Figure 1.7) (Hayden and Ghosh 2008). NFκB orchestrates inflammatory responses of chondrocytes leading to cartilage degradation in OA and directly regulates the expression of catabolic and inflammatory genes including MMP-1, -3 and -13, COX-2, iNOS, IL-6, IL-1 and TNF (Marcu *et al,* 2010).

1.6.5 Matrix Metalloproteinases in OA

During the pathogenesis of OA MMPs are produced by chondrocytes and synovial cells in response to IL-1β stimulation. MMPs are secreted into the ECM where they actively cleave all matrix components including collagens and proteoglycans, resulting in cartilage resorption (Burrage *et al,* 2006). There are a number of MMPs implicated in the pathogenesis of OA and include the collagenases MMP-1, MMP-8 and MMP-13 which degrade interstitial collagens including collagen type I, II and III, the gelatinases MMP-2 and MMP-9 which degrade collagen type IV, the stromelysins MMP-3, -10 and -11 which degrade a broad range of matrix molecules and membrane type I (MT1) MMP-14 (Goldring and Marcu 2009) (Table 1.2).

MMPs in OA	Joint cells	Activity	Substrate	Reference
MMP-1	Chondrocytes, synovial fibroblasts, macrophages	Collagenase	Interstitial Col I, II and III Proteoglycans	Martel-Pelletier *et al,* 1984; Pelletier *et al,* 2003
MMP-2	Chondrocytes, macrophages	Gelatinase	Denatured collagen I IV, V, VII & X, proteoglycans laminin, elastin	Knauper *et al,* 1996; Duerr *et al,* 2004
MMP-3	Chondrocytes, synovial fibroblasts, macrophages, osteoclasts	Stromelysin	Denatured collagen I, II and III, proteoglycans, laminin, elastin pro-MMPs	Unemori *et al,* 1991; Murphy *et al* 2002
MMP-8	Chondrocytes, macrophages	Collagenase	Interstitial Col I, II and II	Burrage *et al,* 2006
MMP-9	Chondrocytes, synovial fibroblasts, macrophages, osteoclast	Gelatinase	Denatured collagen I IV, V, VII & X, proteoglycans laminin, elastin	Murphy *et al,* 2008
MMP-10	Chondrocytes osteoclasts	Stromelysin	Pro-collagenases	Barksby *et al,* 2006
MMP-13	Chondrocytes, synovial fibroblasts, macrophages	Collagenase	Collagen II Proteoglycans	Knauper *et al,* 1996; Murphy *et al,* 2002
MMP-14	Chondrocytes, synovial fibroblast	Membrane-type MMP	Pro-MMPs	Knauper *et al,* 1996; Dreier *et al,* 2004

ADAMTs in OA	Joint cells	Substrate	Reference
ADAMTS-1	Chondrocytes	Proteoglycans	Sandy *et al,* 1991
ADAMTS-4	Chondrocytes, Synovial fibroblasts	Proteoglycans COMP	Nagase *et al* 2002; Tortorella *et al,* 2001; Tortorella *et al,* 1999
ADAMTS-5	Chondrocytes	Proteoglycans	Nagase *et al,* 2002; Tortorella *et al,* 2001
ADAMTS-8	Chondrocytes	Proteoglycans	Collins-Racie *et al,* 2004
ADAMTS-9	Chondrocytes	Proteoglycans	Lin and Liu 2010
ADAMTS-15	Chondrocytes	Proteoglycans	Murphy *et al* 2008
ADAMTS-16	Chondrocytes, Synovial fibroblasts	Proteoglycans	Kevorkian *et al,* 2004; Surridge *et al,* 2006

Table 1.2 The matrix degrading enzymes involved in osteoarthritis pathogenesis.

MMP-1 is a key collagenase involved in the breakdown of ECM components including collagens principally type III but also types II and I in addition to proteoglycans. During OA its activity has been shown to increase in relation to human OA lesions (Pelletier *et al,* 1983; Martel-Pelletier *et al,* 1984) Collagenase MMP-8 primarily cleaves collagen type I (Burrage *et al,* 2006). MMP-3 degrades a broad spectrum of substrates including aggrecan; however it does not have the ability to cleave triple helical collagens (Murphy *et al,* 2002) In addition, MMP-3 has the ability to cleave and activate pro-MMPs including MMP-1 (Unemori *et al,* 1991). *In vivo,* MMP-10 is present in OA synovial fluid and joint tissues, is induced by IL-1β in chondrocytes and synovial cells *in vitro* and activates pro-collagenases (Barksby *et al,* 2006). Collagenase 3 (MMP-13) is a key enzyme involved in collagen II breakdown with the ability to cleave triple helical collagens into ¾ and ¼ fragments (Murphy *et al,* 2002; Knauper *et al,* 1996). MMP-13 also breaks down aggrecan, giving it a dual role in ECM degradation (Burrage *et al,* 2006). MMP-13 displays 5 to 10 times more activity than MMP-1 on collagen type II cleavage; however MMP-1 is expressed at higher levels than MMP-13 (Burrage *et al,* 2006). Both MMP-3 and MMP-13 are expressed at higher levels in RA and OA cartilage and synovial tissue compared to normal joint tissues (Davidson *et al,* 2006; Bau *et al,* 2002; Hembry *et al,* 1995; Okada *et al,* 1992; Wolfe *et al,* 1993; Chubinskaya *et al,* 1999; Yoshihara *et al,* 2000; Koshy *et al,* 2002; Tetlow *et al,* 2001). Both MMP-3 and -8 have been shown to be expressed predominantly in the superficial zone of the cartilage, which may indicate diffusion from the synovial fluid, whilst MMP-13, which is predominantly expressed by chondrocytes, has been identified in the deep zone of the cartilage (Fernandes *et al,* 1998; Moldovan *et al,* 1997). However, in early stage OA denatured and cleaved collagen II epitopes localise with MMP-1 and MMP-13 predominately within the articular surface (Wu *et al,* 2002). MMP-14 is secreted by OA chondrocytes and activates pro-MMP-13, which also cleaves pro-MMP-9 (Dreier *et al,* 2004).

1.6.6 ADAMTSs in OA

Aggrecanases were first described by Sandy *et al* (1991) when IL-1β treatment of bovine articular cartilage was shown to breakdown aggrecan at the Glu^{373}-Ala^{374} bond in the interglobular domain, but not at the Asn^{341}-Phe^{342} MMP site. During OA both ADAMTS-4 and -5 are thought to play an important role in

cartilage degradation and actively cleave proteoglycans (Table 1.2). Studies using both ADAMTS-4 and ADAMTS-5 knockout mice demonstrated that cartilage degradation is prevented following surgical induced joint instability of the meniscus in ADAMTS-5 but not ADAMTS-4 mice, suggesting the former is primarily involved in OA in murine models (Glasson *et al,* 2004; Glasson *et al,* 2005). Interestingly, IL-1β and TNFα induced the expression of ADAMTS-4 but not ADAMTS-5 in human cartilage explants (Tortorella *et al,* 2001). Similarly, in human OA synovium ADAMTS-4 expression was dependant on IL-1β and TNFα however neutralisation of IL-1β or TNFα had no effect on ADAMTS-5 expression, suggesting ADAMTS-5 expression in not dependent on these cytokines in humans (Bondeson *et al,* 2006). In addition ADAMTS-9, -15 and -16 are produced by chondrocytes during OA and ADAMTS-16 levels are elevated in OA cartilage compared to non-arthritic cartilage (Surridge *et al,* 2009; Kevorkian, 2004).

1.6.7 TIMPs in OA

It has been demonstrated that TIMP-1 levels are higher that those of MMPs in non-arthritic cartilage, however during OA there is a decrease in TIMP expression over MMPs, which in part contributes to cartilage breakdown (Dean *et al,* 1989; Martel-Pelletier *et al,* 1994). In addition a decrease in TIMP-1 and -2 expression has been associated with an increase in IL-1β expression as shown in human OA cartilage explants (Martel-Pelletier *et al,* 1994).

1.6.8 Cartilage Degradation

The factors that initiate the onset of cartilage breakdown during OA have not been identified; however some studies suggest that during early stages of OA, aggrecan is initially degraded by matrix degrading enzymes MMP- 3 and ADAMTS-5 followed by an increased activity of collagenases such as MMP-13, which is highly efficient in the cleavage of collagen type II, this is thought to be the point of irreversible cartilage degradation (Loeser *et al,* 2012). Aggrecan is thought to protect collagen from breakdown and thus its breakdown is a pre-requisite for collagen breakdown (Little *et al,* 2007). Collagenases, principally MMP-13, initially cleave the collagen triple helix between Gly775 and Leu776 resulting in the unwinding of the collagen chain, finally other MMPs including MMP-2 and -9 further degrade the collagen molecule (Burrage *et al,* 2006). During proteoglycan breakdown, MMPs cleave the aggrecan core protein at the

Asn341-Phe342 site at the N-terminal end of the interglobular domain between G1 and G2, resulting in the dissociation of G1 from HA and link protein (Fosang *et al,* 1991; Fosang *et al,* 1992). Aggrecan breakdown by ADAMTS involves the proteolytic cleavage at the core protein Glu^{373}-Ala^{374} bond within a conserved region of the interglobular domain, resulting in the separation of G1 (Sandy *et al,* 1991). ECM breakdown products are released from the cartilage and subsequently diffuse into the synovial fluid.

Degradation initially occurs at the articular surface of the superficial zone, progressing into the middle and deep zones of the cartilage with increasing degradation correlating with the Mankin histological grade of the cartilage degeneration with evidence of pericellular degradation in the deeper zones of the cartilage also present with progression of the cartilage lesion (Wu *et al,* 2002; Hollander *et al,* 1995). An increase in MMP synthesis is induced by an increase in pro-inflammatory cytokines namely IL-1β and TNFα, which are produced by the chondrocytes during OA (Mengshol *et al,* 2000; Reboul *et al,* 1996). Furthermore an increase in MMPs over TIMPs during OA is thought to contribute to cartilage breakdown (Martel-Pelletier *et al,* 1994).

1.6.9 Chondrocyte death

Chondrocytes rarely divide in adult articular cartilage and thus there is little cellular turnover. Furthermore, cartilage has no germinal cell layer therefore dead or damaged cells cannot be readily replaced by new cells (Aigner *et al,* 2007). Chondrocyte death is a feature of OA and may occur via apoptosis, necrosis or chondroptosis. Chondrocytes that undergo chondroptosis display features including increased expansion of the endoplasmic reticulum, increase in Golgi apparatus, autophagic vacuoles and extrusion of cellular material into the extracellular space (Roach *et al,* 2004). Furthermore chondrocyte apoptosis has been associated with a decrease in cartilage matrix synthesis and the accumulation of cartilage ECM proteins in the intracellular components of the chondrocytes including the endoplasmic reticulum and the Golgi; these may be attributed to oxidant stress during aging (Yang *et al,* 2005). During OA a combination of these processes is thought to contribute to cartilage breakdown. However, whether apoptosis is a cause or an effect of OA remains to be determined (Zamli and Sharif 2011).

1.6.10 The Synovium and Inflammation in OA

The synovium is composed of a thin layer of cells that are characterised as having pheynotypic features of fibroblasts (Type B synoviocytes) and macrophages (Type A synoviocytes) and are often termed 'fibroblast like cells' (Scanzello and Goldring 2012). Like, chondrocytes, the cells of the synovium also produce lubricin and HA to provide lubrication allowing smooth movement of the joint and maintain the integrity of the articular surface (Hui *et al,* 2012). During OA there is a decrease in both HA and lubricin leading to increased friction at the articular surface, which in part may contribute to cartilage breakdown (Scanzello and Goldring 2012). Synovitis is not only a feature of RA, but also occurs in OA and is thought to contribute to symptoms of OA and increased cartilage degradation (Scanzello and Goldring 2012). Magnetic resonance imaging (MRI) of OA joints has demonstrated the presence of synovial inflammation and is a pathological feature in approximately 50% of OA patients although tends to be more focal in nature (Hayashi *et al,* 2011).

Inflammation of the synovium is a source of pro-inflammatory cytokines, NO, PGE_2 and neuropeptides which all contribute to cartilage degradation (Sellam and Berenbaum 2010; Sutton *et al,* 2009). Other studies have also shown synovitis to be present in early stage OA with an increase in pro-inflammatory cytokines IL-1β and TNFα along with nuclear transcription factors NFκB/RelA and inflammatory mediator COX-2 and increased macrophage infiltration in synovial tissue (Benito *et al,* 2005). Increases in vascular proliferation markers including VEGF, adhesion molecule intercellular adhesion molecule 1 (ICAM-1) and factor VIII have also been identified in synovial tissue derived from patients with early OA (Benito *et al,* 2005). In addition IL-1β also induces the production of the inflammatory mediator PGE_2 by inducing the expression of COX-2 (Amin *et al,* 2000). Both mediators increase the inflammatory reaction impacting on cartilage degradation. Increases of pro-inflammatory cytokines by the inflamed synovium in OA induce the expression of MMPs by chondrocytes (Figure 1.8) therefore it is seen as an important target in the treatment of OA.

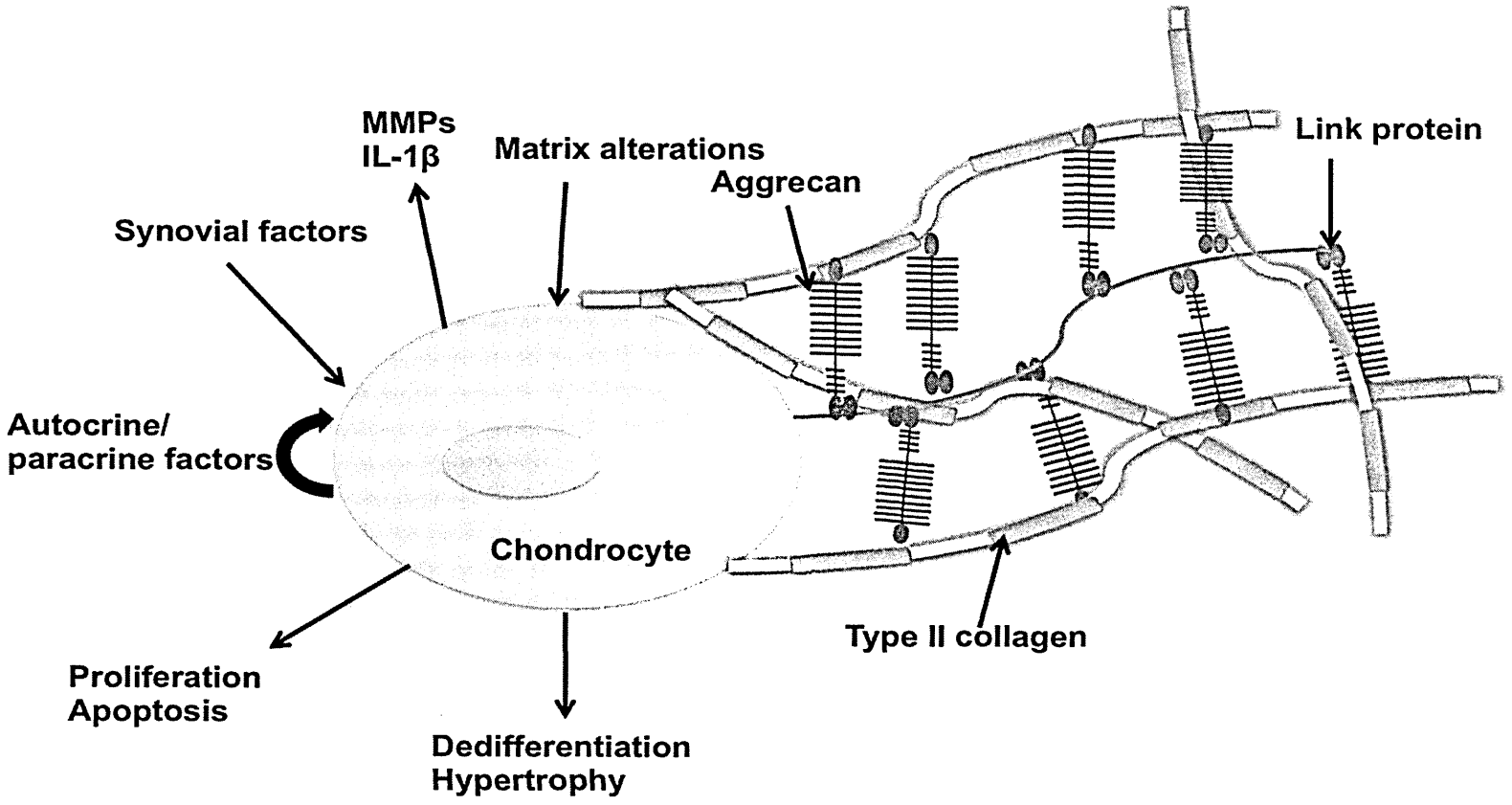

Figure 1.8 Chondrocyte responses in osteoarthritis. During OA chondrocytes are exposed to a number of abnormal external stimuli including synovial factors, autocrine and paracrine factors and ECM alterations induces a plethora of cellular responses including an increase in catabolic factors such as IL-1β and MMP expression. Chondrocytes proliferate and dedifferentiate leading to hypertrophy and apoptosis. The chondrocyte is embedded in an ECM of collagen mainly type II and proteoglycans mainly aggrecan and matrix alteration also lead to the induction of catabolic factors via the activation of integrins by ECM breakdown products. In addition discoidin domain receptors (DDRs) are activated by binding of type II collagen which is exposed on degradation of pericllular matrix.

1.6.11 Chondrocytes in Ageing Cartilage

There is a strong association between ageing and OA. Although during ageing there are changes to the structure of the ECM the development of OA is not always present. During cartilage ageing there is a change in the content of proteoglycans and collagens, changes to the articular surface including softening and surface irregularities and loss of ECM tensile strength and stiffness (Martin and Buckwalter 2002). Changes in the structural organisation of proteoglycan during aging includes a decrease in the molecular size of aggrecan and an increase in matrix protein modification via the formation of advanced glycation end products (AGEs) (Verzijl *et al,* 2003). AGEs are irreversible chemical protein modifications that are known to accumulate with age and in tissue that contain proteins with long half lives such as collagens present in articular cartilage (Verzijl *et al,* 2000). AGEs result in increases in collagen cross-linking, which contributes to the stiffness of the cartilage and also effects the biomechanical and biochemical properties of the tissue (Verzijl *et al,* 2003; Chen *et al,* 2002). Recently, the accumulation of AGEs in chondrocytes has been shown to induce endoplasmic reticulum stress ultimately leading to apoptosis (Yamabe *et al,* 2013). During aging there is a decrease in the chondrocyte's anabolic activities and the cell has limited ability to repair and remodel the ECM, which further diminishes with age. Furthermore there is an increase in the number of senescent chondrocytes during aging which has been associated with a decrease in telomere length (Martin *et al,* 2004).

1.7 Mechanical Loading

Increased load transfer or altered patterns of loading on articular cartilage due to mechanical factors including injury, obesity or joint instability is thought to play a role in the initiation of osteoarthritis (Guilak *et al,* 2004). There are two key mechanisms that can contribute to mechanically induced cartilage breakdown: abnormal loading on normal cartilage or normal loading on abnormal cartilage. However, multiple factors may contribute to abnormal cartilage including aging and genetics, which may predispose the joint to mechanically induced cartilage breakdown (Goldring and Goldring 2007). Studies have shown that dynamic compression increases matrix synthesis, in contrast injurious static compression results in the depletion of proteoglycans in

addition to damage to the collagen network (Guilak *et al,* 2004). Furthermore static compression of cartilage explants induces the expression of matrix degrading genes including MMP-3, -9 and -13 and inflammatory mediators including COX-2 (Fitzgerald *et al,* 2004).

Chondrocytes are able to respond to mechanical stimuli via receptors for ECM components including integrins and discoidin domain receptors (DDR) (Millward-Sadler and Salter 2004). Abnormal loading of the joint leads to activation of the receptors, which in turn initiate the production of matrix degrading enzymes. Information between the ECM and chondrocytes is transmitted via integrins, which are membrane bound receptors expressed by chondrocytes. Integrins respond to mechanical stimuli and bind ECM fragments including collagens and fibronectin (Goldring and Goldring 2007). Activation of TLR induces the production of cytokines including IL-1, IL-6 and TNF and chemokines including IL-8 (Akira and Takeda 2004). Moreover, TLR-2 and TLR-4 are expressed in OA lesion and activation of these receptors was shown to induce catabolism in murine cartilage explants by MMP-3 and -13 (Liu-Bryan and Terkeltaub 2010). DDR-2 receptor expression is increased in OA and the receptors bind collagen type II fibrils, inducing the expression of MMP-13 (Xu *et al,* 2007). These signals provide important information to the chondrocytes; regulating matrix remodelling, cell differentiation, survival and proliferation. Inappropriate activation of integrins during OA can induce the production of matrix degrading enzymes, inflammatory cytokines and chemokines and intracellular pathways leading to cartilage degradation (Shakibaei *et al,* 2008).

1.8 Bone

Bone remodelling occurs during OA at sites of bone damage (Loeser *et al,* 2012). A contributing factor to bone damage and remodelling is thought to be the abnormal mechanical loading of the joint, which occurs during OA. Loss of cartilage is also exacerbated by changes in the bone in OA (Loeser *et al,* 2012). Increased subchondral bone thickening may alter the biomechanical forces on the cartilage causing damage (Goldring and Goldring 2007). Although alteration in the bone occurs in OA, bone mass is maintained, however the altered activities of osteoclasts and osteoblasts result in remodelling of the subchondral and trabecular bone changing its shape and structure (Goldring and Goldring 2010). During OA multinucleated osteoclasts are activated and produce

proteinases including MMP-3, -9, -10, - 12 and cathepsin K which are thought to contribute to bone remodelling and erosions (Murphy and Nagase 2008). Bone remodelling and the formation of new bone occurs at the joint margins and entheseal sites in a process which occurs by endochondral ossification which is thought to recapitulate the cellular mechanism involved in skeletal growth (van der Kraan and van den Berg 2007). Osteophyte formation is a pathological feature of OA formed by the process of inappropriate endochondral ossification and is driven by the production of anabolic factors including transforming growth factor (TGFβ) and bone morphogenetic factor protein 2 (BMP-2) (Zoricic *et al,* 2003; Blaney Davidson *et al,* 2007). Interestingly, there is also evidence to suggest that the formation of osteophytes is a mechanism to restore joint stability (van der Kraan and van den Berg 2007).

The subchondral bone is separated from the articular cartilage by the zone of calcified cartilage and encompasses the tidemark. During disease progression there is notable changes in the calcified cartilage zone. The calcified cartilage progresses into the deep zone of the cartilage with duplication of the tidemark often observed (Loeser *et al,* 2012). Cartilage calcification is driven by the increased production of collagen type X a hypertrophic marker during OA which has been shown to directly correlate with mineralization in patients with late OA (Fuerst *et al,* 2009). In addition, vascular invasion of the cartilage at the osteochondral junction, in part in response to VEGF production by chondrocytes, may provide an additional source of catabolic cytokines and proteinases (Murata et al, 2008; Loeser et al, 2012).

1.9 Pain

The osteochondral junction is thought to be the major source of pain in OA. During OA there is ingrowth of blood vessels which have been shown to localise with nerves which infiltrate from the subchondral bone into the articular cartilage where the tide mark integrity is lost (Walsh *et al,* 2010). These painful stimuli are further exacerbated by the production of nerve growth factors and neurotrophins including: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and substance P by articular chondrocytes, which can be regulated by proinflammatory cytokines (Gigante *et al,* 2003; Grimsholm *et al,* 2008; Forsgren 2009). As the cartilage tissue is avascular and aneural it is important to understand the pain associated with OA, however to date, there is

little knowledge as to what initiates the vascularisation and innervation into the articular cartilage.

1.9.1 Nerve Growth Factor

NGF is a secreted growth factor and a regulator of nociceptive pain and plays a role in the differentiation, development and signalling of neurons (Fiore *et al,* 2009). NGF mediates its effects via two membrane receptors including the low affinity receptor p75 NGFR which shares sequence homology with the TNF receptors and the high affinity receptor transmembrane kinase p140 TrkA (Mallett and Barclay 1991; Kaplan *et al,* 1991). NGF and TrkA expression is increased in human OA chondrocytes compared to normal cells, suggesting that NGF may play a role in the pathogenesis of OA (Iannone *et al,* 2002). There is also evidence to suggest that cytokines are involved in pain signalling during OA as NGF is released by synovial fibroblasts following treatment with IL-1β and TNFα (Manni *et al,* 2003). The role of NGF in OA pain has recently been demonstrated; sensory nerve fibres expressing NGF were found associated with blood vessels in osteochondral angiogenesis (Walsh *et al,* 2010). Furthermore, subchondral bone marrow replacement by fibrovascular tissue expressing VEGF was associated with an increased in NGF (Walsh *et al,* 2010). These findings suggest that that osteochondral angiogenesis and NGF may be associated with OA pain.

1.9.2 Substance P

Substance P is a neuropeptide involved in pain signalling and is secreted by nerves where it acts as a neurotransmitter and neuromodulator and by inflammatory cells acting as a proinflammatory mediator (O'Connor *et al,* 2004). Substance P mediates its effects via binding to the neurokinin-1 receptor (NK1R) and is thought to be associated with the progression of OA as infusion of substance P into the knee joint in animal models of arthritis increased the severity of arthritis (Levine *et al,* 1984). Furthermore IL-1β has been shown to increase substance P levels in human chondrocytes (Im *et al,* 2008) suggesting that IL-1β plays a role in pain signalling in cartilage degeneration.

1.10 Genetic Factors

There is evidence to suggest that genetic factors are involved in the pathogenesis of OA. Genome wide association studies have identified

polymorphisms and mutations in genes encoding ECM and signalling molecules including IL-1 gene cluster, IL-4 receptor alpha chain (IL4R), frizzled-related protein 3, aspirin (ASPN) and collagen type II (COL2AI) (Loughlin 2005; Valdes *et al,* 2007). More recently loci associated with the risk of developing OA have been identified and include genes involved in the regulation of body weight (Valdes *et al,* 2007;arcOGEN Consortium *et al,* 2012). Thus there is increasing evidence to suggest that gene defects may contribute to the early onset of OA (Goldring and Goldring 2007).

1.11 Epigenetics

Epigenetic changes are thought to play a role in the development of arthritis. Studies have shown that DNA methylation, histone modification and microRNAs (miRNAs) all play a role in cartilage degradation during OA (Reynard and Loughlin 2012) In late OA, reduced methylation of specific CpG islands within the promoters of MMP-13 and ADAMTS-4 has been demonstrated leading to an increased expression of these genes due to hypomethylation (Roach *et al,* 2005; da Silva *et al,* 2009; Cheung *et al,* 2009). miRNAs are a family of non-coding RNAs that bind to target mRNA and decrease or inhibit the expression of genes. A number of miRNAs are differentially expressed in OA and normal cartilage (Reynard and Loughlin 2012). miR-146a is increased by IL-1β in human OA cartilage, however it was also demonstrated that miR-146a may decrease IL-1β induced MMP-13 expression via downregulation of IRAK1 and TRAF6, suggesting that miR-146a is a negative feedback regulator in OA cartilage (Yamasaki *et al,* 2009). *In vivo* studies have shown that targeted deletion of miR-146a in mice resulted in age-related OA phenotypes and mice over expressing miR-146a were resistant to antigen-induced arthritis (Miyaki *et al,* 2010).

1.12 Arthritis Therapies

1.12.1 RA Therapies

A number of disease modifying anti-rheumatic drugs (DMARDs) have been used in the treatment of RA, which include cytotoxic, immunosuppressive and antimalarial drugs (Doan and Massarotti 2005). Currently early aggressive treatment of RA with DMARDs is used, often deploying methotrexate alone or in combination with other DMARDs such as sulphasalazine and or

hydroxychloroquine (Sokka *et al,* 2008; Gaujoux-Viala *et al,* 2010). Although there are therapies that are effective in targeting structural damage associated with RA, there are no current therapies to prevent the disease progression in OA and ultimately patients often undergo total joint replacements.

Biological therapies have developed as a result of increasing knowledge of the pathogenesis of RA. They target proinflammatory cytokines, principally TNF, IL-1 and IL-6 and also T and B cells that are involved in the disease processes. TNF inhibitors include infliximab, a chimeric monoclonal antibody to TNF, etanercept, a soluble TNF receptor construct and adalimumab, a humanised monoclonal antibody to TNF (Tak and Kalden 2011). Anakinra, a recombinant human IL-1 receptor antagonist, is used to inhibit IL-1, though is not always as effective as the TNF inhibitors due to systemic application (Mertens and Singh 2009) and tocilizumab a humanised IL-6 receptor antibody to inhibit IL-6 (Tak and Kalden 2011; Nurmohamed 2009). Newer biological therapies target B and T cells and include rituximab which is a chimeric monoclonal antibody to CD20 expressed on B cells and abatacept which is an anti-cytotoxic T lymphocyte antigen 4 (CTLA-4) antibody which blocks the activation of T cells (Tak and Kalden 2011; Nurmohamed 2009). In addition, non-steroidal inflammatory drugs (NSAIDs) and glucocorticoids may be used to help control inflammation (Doan and Massarotti 2005). These may be used in both RA and OA.

1.12.2 Anti-Cytokine OA Therapies

The success of some of the anti-cytokine therapies in RA has prompted investigation of their effectiveness in OA. There have been a number of anti-cytokine therapies tested in clinical trials and animal models of OA. Anti-IL-1 therapies tested include: Anakinra, a recombinant IL-1Ra and IL-1Ra gene therapy using retroviral or adenoviral vectors. *In vivo,* intra-articular injections of IL-1Ra protected against the development of cartilage lesions in OA, in part by a reduction in MMP-1 expression (Caron *et al,* 1996). In clinical trials intraarticular injections of recombinant human IL-1Ra in patients with knee OA was well tolerated with no acute inflammatory reaction, however in a 12 week randomised trials Anakinra failed to provide effective relief from knee OA symptoms compared to placebo controls suggesting that IL-1Ra is ineffective in treating OA (Chevalier *et al,* 2009) IL-1Ra gene therapy delivered by

intraarticular injections in animals models of OA reduced the severity of cartilage lesions, disease progression and activity (Pelletier *et al,* 1997; Fernandes *et al,* 1999; Frisbie *et al,* 2002; Zhang *et al,* 2004). Furthermore anti-inflammatory drug licofelone displayed cartilage protective effects and reduced the volume of cartilage loss in patients with symptomatic OA (Raynauld *et al,* 2009). Anti-TNF therapies include Adalimumb and Infliximab both are monoclonal anti-TNF antibodies. In clinical trials patients with hand OA treated with Adalimumb failed to display signs of improved OA symptoms (Magnano *et al,* 2007). However, intra-articular injections of Infliximab reduced pain symptoms and lesion progression in patients with hand OA whilst displaying no systemic adverse reactions (Fioravanti *et al,* 2009).

Development of disease modifying drugs for the treatment of osteoarthritis (disease modifying anti osteoarthritic drugs (DMAODs)) is not as advanced as for RA. Treatments tend to target pain and inflammation rather than the underlying disease mechanisms. They include the use of NSAIDs and COX-2 inhibitors and intra-articular injections of corticosteroids and hyaluronic acid. Nutraceuticals such as glucosamine sulphate are also in common usage but their efficacy is uncertain (Goldring 2006). There is therefore a need to develop effective DMAODs, targeting key events such as cartilage breakdown, which may involve inhibiting cytokine actions, informed by use of these therapies in RA, or actions of catabolic enzymes such as selected MMPs and ADAMTSs (Murphy and Nagase 2008; Goldring 2006).

1.12.3 Targeting MMPs and ADAMTS

The inhibition of MMPs is an attractive target for OA therapy, since IL-1β and TNFα increase the expression of a number of MMPs involved in the pathogenesis of OA. However, the use of broad-spectrum MMP inhibitors has had little success, due to their toxicity caused by non-selectivity of MMP inhibition (Murphy and Nagase 2008; Krzeski *et al,* 2007; Tu *et al,* 2008). Other, more specific inhibitors of MMPs are now being investigated, that have different Zn-binding groups (Tu *et al,* 2008; Clutterbuck *et al,* 2009). The main target for MMP inhibition is MMP-13 as it is predominantly up-regulated in OA cartilage and has the ability to cleave triple helix collagens and aggrecan (Dahlberg *et al,* 2000). *In vivo* studies have shown that inhibition of MMP-13 using a highly

selective inhibitor reduced cartilage degradation without inducing fibroplasias in a rat model of musculoskeletal syndrome side effects (Johnson *et al,* 2007). The inhibition of ADAMTS-4 is another possible therapeutic target for preventing cartilage breakdown in OA, and unlike MMPs; ADAMTSs have a narrower substrate selectivity (Tortorella *et al,* 2009).

1.12.4 Targeting OA Signalling Pathways

Inhibition of IL-1β signalling pathways is another potential target for OA therapies. Since NFκB activation regulates the expression of cytokine and chemokines, inflammatory mediators and matrix degrading enzymes in OA, inhibition of this pathway may be of therapeutic value in the treatment of OA (Marcu *et al,* 2010). The inhibition of NFκB in animal models of RA was shown to reduce inflammation and cartilage breakdown, however the significance of this in OA models has yet to be determined (Marcu *et al,* 2010). Furthermore other signalling pathways including MAPK may also provide new therapeutic targets in the treatment of OA (Kapoor *et al,* 2011).

1.13 Cannabinoids

Cannabis, from the plant *Cannabis sativa,* has been used medicinally and recreationally for many years because of its anti-inflammatory, analgesic and psychoactive properties (Joy *et al,* 1999). It is the source of 60 different pharmacologically active cannabinoids of which Δ^9-tetrahydrocannabinol (Δ^9-THC) is the major psychoactive component. Other phytocannabinoids include Δ^8-tetrahydrocannabinol (Δ^8-THC), a weak psychoative cannabinoid cannabinol (CBN) and non-psychoactive cannabidiol (CBD) and cannabigerol (CBG) (Pertwee *et al,* 2010). In addition to phytocannabinoids synthetic cannabinoids have been developed as research tools and include (-)-11-hydroxy-Δ^8-THC-demethylheptyl (HU210), CP55,940 and R(+)WIN 55, 212-2 (WIN-55). Lastly, endogenous cannabinoids (endocannabinoids) are produced by mammalian tissues and include anandamide (arachidonoylethanolamide (AEA)) and 2-arachidonoylglycerol (2-AG) (Pertwee 2005; Pacher *et al,* 2006). Cannabinoids produce their effects by binding to and activating receptors found in cell membranes or intracelluarly. The two classical cannabinoid receptors include cannabinoid receptor 1 and 2 (CB1 and CB2) (Matsuda *et al,* 1990; Munro *et al,* 1993).

1.13.1 Classical Cannabinoids

The classical group of cannabinoids includes both phytocannabinoids and synthetic cannabinoids and are dibenzopyran derivatives (Pertwee *et al,* 2010; Howlett *et al,* 2002). Classical cannabinoids include the phytocannabinoids Δ^9-THC, Δ^8-THC and synthetic cannabinoid HU210. Δ^9-THC binds both CB1 and CB2 receptors with similar affinity with Kis of 5.05 to 80.3 nM and 3.13 to 75.3 nM respectively and also acts as a partial agonist at these receptors (Pertwee *et al,* 2010). HU210 is a potent agonist at both CB1 and CB2 and has higher binding affinity than THC and displays long-lasting *in vivo* pharmacological effects with Kis of 0.06 to 0.73 nM and 0.17 to 0.52 mM receptively (Pertwee *et al,* 2010).

1.13.2 Non-Classical Cannabinoids

Non-classical cannabinoids have a similar structure to the classical group of cannabinoids and are analogues of Δ^9-THC but lack a pyran ring (Pertwee *et al,* 2010). CP55,940 belongs to the non-classical group of cannabinoids and binds CB1 with a Ki of 0.5 to 5 nM and CB2 with a Ki of 0.69 to 2.8 nM (Pertwee *et al,* 2010).

1.13.3 Aminoalkylindole

WIN-55 is an aminoalkylindole and is widely used in cannabinoid research and does not share the same structure as other cannabinoids. WIN-55 binds to the classical receptors CB1 with a Ki of 1.89 to 123 nM and is thought to display a slightly greater affinity for CB2 with a Ki of 0.28 to 16.2 nM (Pertwee *et al,* 2010).

1.13.4 Endocannabinoids

Mammalian cells can synthesise and secrete endogenous cannabinoids which activate cannabinoid receptors (Pertwee 2006). Endocannabinoids are a sub group of eicosanoids and include AEA and 2-AG (Burstein and Zurier 2009). The endocannabinoid system is thought to be immunomodulatory and also autoprotective (Pertwee 2005). AEA binds to both CB1 and CB2 receptors, although has a slightly higher affinity for CB1 (Pertwee 2006). Similarly, 2-AG has affinities for both CB1 and CB2 receptors (Pertwee 2006). Other endogenous cannabinoids include 2-arachidonolyglycerol ether (noladin ether), O-arachidonoyl ethanolamine (virodhamine), N-dihomo-γ-linolenoyl ethanolamine, N-docosatetraenoyl ethanolamine, oleamide, N-arachidonoyl

dopamine (NADA) and N-oleoyl dopamine (OLDA) (Pertwee *et al,* 2010). Other structurally related endogenous fatty acid compounds which do not bind to the classical cannabinoid receptors but are thought to facilitate the actions of endocannabinoids and modulate the endocannabinoid system include oleoyl ethanolamide (OEA) and palmitoyl ethanolamide (PEA) (Mechoulam *et al,* 1998;Lambert and Di Marzo 1999). The endocannabinoid receptor system includes receptors CB1 and CB2, endogenous ligands and the enzymes that breakdown endogenous cannabinoids (Mackie and Stella 2006). The production of endogenous cannabinoids is tightly regulated, they are synthesised on demand in response to elevated intracellular calcium and can act in an autocrine manner intracellularly or are secreted from the cell and act in an autocrine or paracrine fashion (Di Marzo *et al,* 1999; Piomelli *et al,* 1998). They are rapidly broken down by intracellular endogenous cannabinoid degrading enzymes including fatty acid amide hydrolase (FAAH), which readily degrades AEA, and monoacylglycerol lipase (MAGL), which degrades 2-AG (Dinh *et al,* 2002; Deutsch *et al,* 2002).

1.14 Cannabinoid receptor 1 and 2

CB1 and CB2 are G-protein coupled receptors (GPCRs) with an extracellular N-terminal and an intracellular C-terminal domain with seven transmembrane hydrophobic alpha chains. CB1 receptors are expressed in the central nervous system and are associated with decreasing neuronal excitability and are known to mediate the psychoactive effects of cannabinoids such as THC (Howlett *et al,* 2002). CB2 receptors are mainly expressed in immune cells (Howlett 2002) where they have been found to modulate cytokine release and are associated with a decrease in immune cell function (Pertwee 2006). Not all the physiological effects observed with cannabinoid ligands, both exogenous and endogenous are mediated by CB1 and CB2 receptors. As well as the two classical cannabinoid receptors there is evidence to suggest that cannabinoids can produce their effects via other receptors including G protein-coupled receptor 55 (GPR55), G protein-coupled receptor 18 (GPR18), transient receptor vanilloid 1 (TRPV1) and peroxisome proliferator activated receptors alpha and gamma (PPARα and γ) (Figure 1.9). Phytocannabinoids: CBN and CBD and endogenous cannabinoids OEA and PEA, which are structural analogues of AEA, have no binding affinity to CB1 or CB2 (Brown 2007)

suggesting that these cannabinoids mediate their effects via a non-CB1/CB2 receptor.

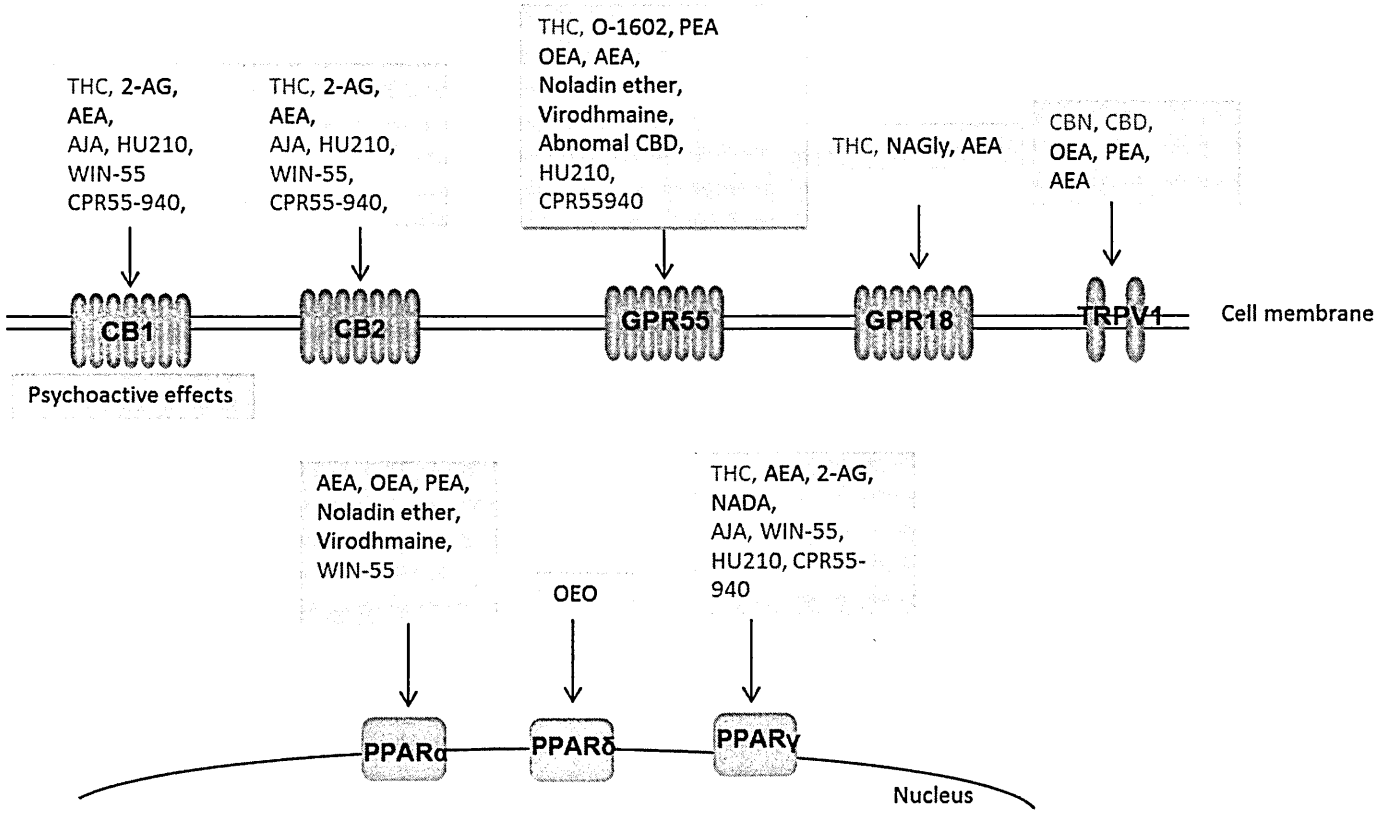

Figure 1.9 Cannabinoid receptors and the cannabinoids that activate them. Synthetic cannabinoid agonists are shown in red, endogenous cannabinoid agonists shown in black and phytocannabinoids shown in blue. Cannabinoid receptor 1 and 2 (CB1 and 2), G protein coupled receptors 55 and 18 (GPR55 and 18), transient receptor potential vanilloid type I (TRPV1), peroxisome proliferator activated receptors alpha, delta and gamma (PPARα, δ and γ). Ajulemic acid (AJA), Tetrahydrocannabinol (THC), anandamide (arachidonoylethanolamide (AEA)), 2-arachidonoylglycerol (2-AG), cannabinol (CBN), cannabidiol (CBD), oleoylethanolamide (OEA), palmitoylethandamide (PEA), N-arachidonoyl dopamine (NADA), N-Arachidonylglycine (NAGly)

1.14.1 CB1 and CB2 Receptor Signalling

CB1 and CB2 receptors are coupled through $G_{i/o}$ proteins (Gi1, 2 and 3 and Go1 and 2) at their intracellular surface and ligands that bind these receptors activate transducing G-proteins (Howlett 2005). Following ligand binding, signal transduction via G-proteins inhibits adenylyl cyclase, causing decreased intracellular cyclic AMP and increases intracellular Ca^{2+}. CB1 is involved in the control of neurotransmission via the modulation of ion channels (Figure 1.10) (Howlett 2005). In addition activation of cannabinoid receptors leads to the phosphorylation and activation of MAPKs including ERK1/ERK2, p38 and JNK signalling pathways, resulting in the regulation of nuclear transcription factors (Figure 1.10). Pertussis toxin is used to study the activation of GPCRs via preventing the interaction of G proteins, thus inhibiting the induction of intracellular signalling pathways following ligand binding (Howlett 2005).

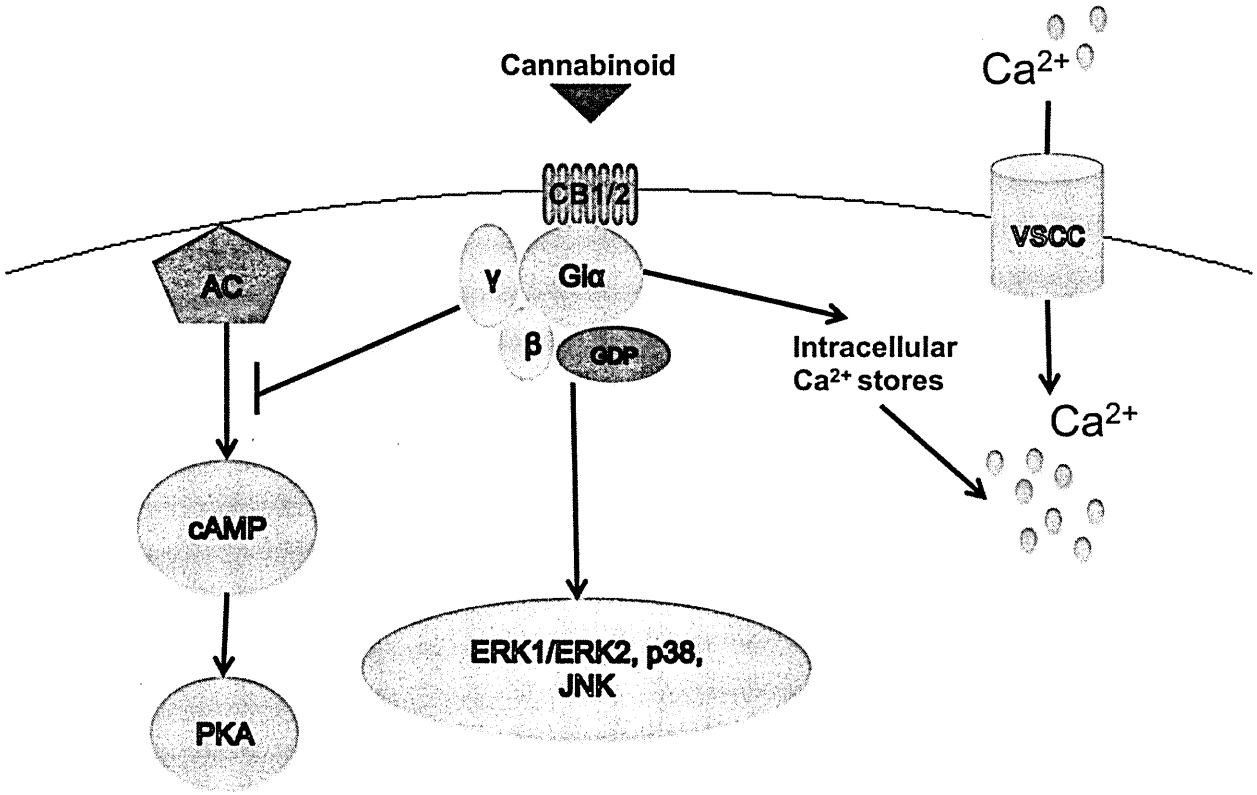

Figure 1.10 Classical cannabinoid receptor signaling pathway. Cannabinoids mediate their effects via binding to specific G protein coupled receptors. Cannabinoid ligands activate the classical cannabinoid receptors CB1/CB2 and induce the inhibition of the adenylyl cyclase (AC) cyclic AMP-protein kinase A (PKA) pathway, the Inhibition of voltage sensitive Ca2+ channels (VSCC) and the induction of Ca^{2+} release from intracellular stores and the activation of the mitogen activated protein kinases (MAPK) signaling pathway including extracellular-signal-regulated kinase (ERK), c-Jun-N-terminal kinases (JNK) and p38.

1.14.2 G-protein coupled receptor 55

The orphan G-protein coupled receptor GPR55 has been identified as a cannabinoid receptor, however it has low sequence homology with CB1 and CB2 (McPartland *et al,* 2006; Sharir and Abood 2010). GPR55 lacks the classical cannabinoid binding pocket that is present in both the CB1 and CB2 receptors (Petitet *et al,* 2006). Recent studies have shown that the GPR55 binding pocket consists of many hydrophilic residues, which is in contrast to the CB1 and CB2 receptor's highly hydrophobic cannabinoid binding pocket (Kotsikorou *et al,* 2011). The structure of the GPR55 consists of a deep vertical and highly hydrated binding pocket that allows ligands to bind vertically (Kotsikorou *et al,* 2011). In contrast to the CB1 and CB2 receptors, the third extracellular loop of GPR55 is longer and is composed of many charged amino acids (Kotsikorou *et al,* 2011). Together these findings suggest that GPR55 is structurally distinct from CB1 and CB2 and thus may bind distinct cannabinoid ligands.

Interest in GPR55 as a cannabinoid receptor was first recognised by GlaxoSmithKline when it was expressed in yeast (Brown 2001). Interestingly the CB1 antagonist AM251 activated GPR55 acting as an agonist for this receptor (Brown 2001). GPR55 was also expressed in HEK_{293} cells and cannabinoids including endogenous, synthetic and natural, stimulated binding of GTPγS to the receptor (Drmota E 2004). GPR55 has now been found to bind a number of cannabinoid ligands (Ryberg *et al,* 2007) (Figure 1.9). Endocannabinoids AEA, PEA, OEA, 2-AG, virodhamine norladin ether, the natural cannabinoids THC and abnormal CBD and synthetic cannabinoids: CPR55940, O-1602, HU210 and AM251 a CB1 antagonist which also activates GPR55, bind to GPR55 stimulating GTPγS binding (Ryberg *et al,* 2007). However synthetic cannabinoids WIN-55 and CB1 antagonist AM281 are inactive at GPR55 and CBD antagonized the agonist effects of CPR55940. In a more recent study, β-arrestin green fluorescent chimeras were used to study GPR55 agonists and antagonists. These are intracellular proteins which bind directly to activated GPCRs and desensitize them enabling a fluorescent signal to be detected from the receptor-arrestin complex (Kapur *et al* 2009). In this study AM251 and rimonabant (SR141716A), CB1 and CB2 antagonists, were shown to acts as GPR55 agonists activating protein kinase signalling. However CP55940 acted

as an antagonist and partial agonist preventing the internalization of GPR55 (Kapur *et al,* 2009). Studies propose that GPR55 is a cannabinoid receptor which produces responses different from those of CB1, and CB2 receptors. However there is conflicting data which suggests GPR55 may not be a specific cannabinoid receptor. It has been shown that a number of endogenous and synthetic cannabinoid ligands do not activate GPR55 and suggested that lysophosphatidylinositol (LPI) is the natural endogenous ligand of GPR55 receptors (Oka *et al,* 2007). Interestingly, similar to CB1 and CB2 induced intracellular signalling pathways GPR55 activation induces the phosphorylation of MAPKs including ERK1/ERK2 and p38 and the release of Ca^{2+} stores (Nevalainen and Irving 2010; Oka *et al,* 2010). Although not clear cut, overall evidence suggests that GPR55 interacts with different cannabinoid ligands and may activate signalling pathways other than those induced by CB1 and CB2.

1.14.3 G-protein coupled receptor 18

GPR18 has been identified as a receptor for cannabinoids and was initially cloned from a human T-cell line (Kohno *et al,* 2006). Cannabinoid ligands, which have been shown to be full agonists at GPR18, include endogenous cannabinoid anandamide, N-Arachidonylglycine (NAGly), the metabolite of anandamide and phytocannabinoid THC (Figure 1.9). In addition phytocannabinoid CBD was shown to have low binding affinity at GPR18 (McHugh *et al,* 2012). Interestingly, both AEA and THC were both shown to be partial agonists at CB1 and have low binding affinity to CB2 (Pertwee *et al,* 2010) as determined by the activation of GPR18 heterologously expressed in HEK293 cells producing phosphorylation of ERK1/ERK2. Furthermore, NAGly induced release of Ca^{2+} stores and inhibited cyclic adenosine 3', 5'-monophosphate (cAMP) formation, effects that were shown to be pertussis toxin sensitive (Kohno *et al,* 2006). Although sharing structural similarity to anandamide, NAGly has been shown to be inactive at both CB1 and CB2 receptors (Sheskin *et al,* 1997). Under physiological conditions, NAGly initiates microglial migration in the central nervous system via GPR18 activation (McHugh *et al,* 2012; McHugh 2012). GPR18 has little primary sequence homology with CB1 and CB2 receptors, suggesting that distinct cannabinoid ligands bind and activate it.

1.14.4 Transient Receptor Potential Vanilloid 1

TRPV1 is an ion channel linked receptor composed of six transmembrane domains with cytosolic C- and N- terminal domains and a pore forming hydrophobic region between the fifth and sixth transmembrane domains (Tominaga and Tominaga 2005). TRPV1 is involved in peripheral nociception and is activated by capsaicin in addition to heat, acid and lipids (Tominaga and Tominaga 2005). TRPV1 is primarily expressed by sensory neurons and acts as an endogenous cannabinoid receptor for AEA and has also been shown to bind phytocannabinoids, including CBD (Figure 1.9) (Bisogno *et al,* 2001; Smart and Jerman 2000).

1.14.5 Peroxisome Proliferator-Activated Receptors (PPARs)

Cannabinoids are also thought to activate PPARs. PPARs are ligand-activated nuclear transcription factors which on activation heterodimerise with the retinoid X-receptor (RXR) and bind to the PPAR response elements of target genes involved in regulation of metabolism, particularly lipid metabolism, cell differentiation and inflammation (O'Sullivan and Kendall 2010; Desvergne and Wahli 1999). There are three main isoforms of PPAR including PPARα, δ and γ of which PPARγ is best characterised for its anti-inflammatory effects and is most extensively studied. PPARγ has two isoforms PPAR-γ1 and PPAR-γ2 formed by alternative splicing and promoter usage (Fajas *et al,* 1997). All subtypes of PPARs are widely expressed, particularly in the central nervous system (CNS) and peripheral nervous system (O'Sullivan 2007).

PPARs have been shown to be activated by a number of cannabinoids including the synthetic cannabinoid ajulemic acid (AJA), which has been shown to bind to the ligand binding domain of human PPARγ (Ambrosio *et al,* 2007). Furthermore, endogenous cannabinoids AEA, 2-AG and NADA, phytocannabinoid Δ^9-THC and synthetic cannabinoids WIN-55, CP55,940, HU210 have been shown to bind to and activate PPARγ receptors (O'Sullivan and Kendall 2010; O'Sullivan 2007; Bouaboula *et al,* 2005) (Figure 1.9). Endogenous cannabinoid AEA, OEA, PEA, noladin ether and virodhamine and synthetic cannabinoid WIN-55 display PPARα binding and promote transcriptional activity (Sun *et al,* 2006; Lo Verme *et al,* 2005; Fu *et al,* 2003)

and OEO increased transcriptional activity of PPARδ (Fu *et al,* 2003) (Figure 1.9).

1.15 Cannabinoid Receptor Expression in Joint Cells

The expression of cannabinoid receptors in joint cells has been identified (Table 1.3) and there is evidence to suggest that activation of these receptors by cannabinoid and non-cannabinoid ligands may display chondroprotective and anti-inflammatory effects.

1.15.1 Cannabinoid Receptor 1 and 2

CB1 and CB2 receptors have been shown to be expressed in bovine articular chondrocytes (Mbvundula *et al,* 2006) and have been detected in the synovium and fibroblast like synovial cells of OA and RA patients and human chondrocytes (Richardson *et al,* 2008; Andersson *et al,* 2011). Furthermore, CB1 and CB2 are expressed by bone cells including osteoclasts, osteoblasts and osteocytes and are thought to play a role in bone metabolism as demonstrated by *in vivo* knockout mice studies (Idris and Ralston 2010).

1.15.2 G Protein-Coupled Receptor 55

Recently GPR55 expression has been demonstrated in both normal and OA human chondrocytes, however its role in chondrocyte metabolism is unknown (Andersson *et al,* 2011). Furthermore GPR55 has been shown to be expressed in human and mouse osteoclasts and osteoblasts and activation of GPR55 with cannabinoid CBD which is known to act as an antagonist at this receptor, was shown to regulate osteoclast number and function (Whyte *et al,* 2009).

1.15.3 G Protein-Coupled Receptor 18

Currently there is no published data on the expression of GPR18 in cells of the joint. GPR18 is primarily expressed in the testes and spleen and other tissues and cells involved in endocrine and immune functions including, peripheral blood leukocytes, the small intestine and the thymus (Gantz *et al,* 1997).

1.15.4 Transient Receptor Potential Vanilloid 1

TRPV1 expression has been identified in human OA chondrocytes and OA and RA synovial fibroblasts (Gavenis *et al,* 2009; Engler *et al,* 2007). Furthermore, TRPV1 is expressed by bone tissue and human osteoclast cultures (Rossi *et al,* 2009).

1.15.5 Peroxisome Proliferator Activated Receptors

PPARγ has been shown to be expressed in rat chondrocytes as well as in human chondrocytes and human synovial fibroblasts (Bordji *et al,* 2000; Shao *et al,* 2005; Fahmi *et al,* 2001; Fahmi *et al,* 2002). PPARγ has been found to be mainly located in superficial zone of human cartilage tissue and studies also suggest that these receptors are involved in the modulation of cartilage metabolism in arthritic diseases as protein expression of PPARγ was significantly lowered in human OA cartilage compared to normal human cartilage and was also decreased during the OA progression in animal models (Afif *et al,* 2007; Nebbaki *et al,* 2013). Recently studies have demonstrated that PPARγ knockout mice develop OA, suggesting that PPARγ plays an important role in normal ECM turnover (Vasheghani *et al,* 2013). Furthermore IL-1β has been shown to down-regulate the expression of PPARγ in human OA chondrocytes and rat synovial fibroblasts (Afif *et al,* 2007; Moulin *et al,* 2005; Boyault *et al,* 2001).

PPARα is expressed by human OA chondrocytes, rat, dog and guinea pig chondrocytes and rat synovial fibroblasts (Bordji *et al,* 2000; Shao *et al,* 2005; Afif *et al,* 2007; Nebbaki *et al,* 2013; Moulin *et al,* 2005; Clockaerts *et al,* 2011). Similarly, PPARδ is expressed by human OA chondrocytes, rat, dog and guinea pig chondrocytes and rat synovial fibroblasts (Bordji *et al,* 2000; Shao *et al,* 2005; Afif *et al,* 2007; Nebbaki *et al,* 2013; Moulin *et al,* 2005). All three subtypes of PPARs are expressed by bone cells (Giaginis *et al,* 2007).

.

Joint cell type/tissue	Cannabinoid Receptor Expressed	Species	Reference
Cartilage/ Chondrocytes	CB1	Human & bovine	Andersson *et al,* 2011; Mbvundula *et al,* 2006
	CB2	Human & bovine	Andersson *et al,* 2011; Mbvundula *et al,* 2006
	GPR55	Human	Andersson *et al,* 2011
	PPARα	Human, murine, guinea pig & dog	Clockaerts *et al,* 2011; Afif *et al,* 2007; Bordji *et al,* 2000, Boyault *et al,* 2001; Nebbaki *et al,* 2013
	PPARδ	Human murine, guinea pig & dog	Afif *et al,* 2007; Shao *et al,* 2005; Nebbaki *et al,* 2013,
	PPARγ	Human murine, guinea pig & dog	Fahmi *et al,* 2001; Shan *et al,* 2004; Afif *et al,* 2007; Bordji *et al,* 2000; Boyault *et al,* 2001; Shao *et al,* 2005; Nebbaki *et al,* 2013;
	TRPV1	Human	Gavenis *et al,* 2009
Synovial tissue/ synovial like-fibroblasts	CB1	Human	Richardson *et al,* 2008; Selvi *et al,* 2008
	CB2	Human	Richardson *et al* 2008, Selvi *et al* 2008
	PPARα	Murine	Moulin *et al,* 2005
	PPARδ	Murine	Moulin *et al,* 2005
	PPARγ	Human & murine	Fahmi *et al,* 2001, Moulin *et al* 2005,
	TPRV1	Human	Engler *et al,* 2007
Bone cells	CB1	Human & murine	Whyte *et al,* 2012; Rossi *et al,* 2009; Idris *et al,* 2005; Ofek *et al,* 2006; Idris *et al,* 2009
	CB2	Human & murine	Whyte *et al* 2012; Rossi *et al,* 2009; Idris *et al* 2005; Ofek *et al* 2006; Idris *et al,* 2009
	GPR55	Human and murine	Whyte *et al,* 2009
	PPARα	Murine	Giaginis *et al,* 2007
	PPARδ	Murine	Giaginis *et al,* 2007
	PPARγ	Murine	Giaginis *et al,* 2007
	TPRV1	Human	Rossi *et al,* 2009

Table 1.3 Cannabinoid Receptor Expression in Joint Cells

1.15.6 Cannabinoids and Cannabinoid Receptor Activation in OA

Cannabinoids are produced by a number of cell types including, bone and synovial cells (Richardson *et al,* 2008; Buckley 2008). In view of their analgesic, anti-inflammatory and immunomodulatory properties (Mbvundula *et al,* 2004; Croxford and Yamamura 2005; Klein 2005), the effects of cannabinoids have been studied in animal models of arthritis. Synthetic cannabinoid AJA, a synthetic derivative of Δ^9-THC, reduced inflammation and the severity of adjuvant-induced arthritis (Zurier *et al,* 1998). Non-psychoactive cannabinoid: cannabidiol reduced joint damage and inflammation in murine collagen induced arthritis in a dose-dependent manner (Malfait *et al,* 2000). Similarly, HU-320 a metabolite of a synthetic homologue of cannabidiol reduced joint damage in collagen-induced arthritis (Sumariwalla *et al,* 2004). Together these studies suggest that cannabinoids have potential as therapeutic agents for arthritis.

In vitro studies have also demonstrated the anti-inflammatory and potential chondroprotective effects of cannabinoids. AJA reduced IL-1β production in lipopolysaccharides (LPS) stimulated human peripheral blood mononuclear cells and synovial monocytes and IL-1α or TNF-stimulated release of MMP-1, -3 and -9 from fibroblast-like synovial cells (Johnson *et al,* 2007;Zurier *et al,* 2003). It has been shown that synthetic cannabinoids CP55, 940 and WIN-55 reduced IL-6 and IL-8 production in IL-1β-stimulated human RA and OA fibroblast-like synovial cells and (Selvi *et al,* 2008). Furthermore, synthetic cannabinoids WIN-55 and HU210 prevented IL-1α-stimulated proteoglycan and collagen breakdown in bovine nasal cartilage explants suggesting direct effects on chondrocytes (Mbvundula *et al,* 2006). In addition, WIN-55 also inhibited IL-1α-stimulated production of NO and PGE_2 production, the induction of iNOS and COX-2 expression and NF_KB activation in bovine articular chondrocytes (Mbvundula *et al,* 2006; Mbvundula *et al,* 2005). Cannabis-based therapies have also been trialled for relief of pain in RA and were shown to reduce disease activity (Blake *et al,* 2006).

GPR55 receptor activation with O-1602 a synthetic cannabinoid related to abnormal cannabinoid cannabidiol was found to reduce nociception in a rat model of arthritis (Schuelert and McDougall 2011). In addition O-1602 has also

been found to inhibit osteoclast formation *in vitro* in a mouse model (Whyte *et al,* 2009).

TRPV1 is thought to be associated with OA pain as shown by TRPV1 knockout mice, which have reduced thermal hyperalgesic sensitivity in an adjuvant-induced arthritis model (Keeble *et al,* 2005). Cannabinoid CBD demonstrated anti-inflammatory effects in a rat model of acute inflammation, effects that were thought to be mediated by TPRV1 (Costa *et al,* 2004). Furthermore, TRPV1 is thought to activate osteoclasts thus inducing bone resorption (Rossi *et al,* 2009).

There is growing evidence to suggest that activation of PPARs with both cannabinoid and non-cannabinoid agonists may be a potential target for the treatment of OA and RA via preventing catabolic pathways (O'Sullivan and Kendall 2010; Fahmi *et al,* 2001; Fahmi *et al,* 2002; Johnson *et al,* 2007; Clockaerts *et al,* 2011; Fahmi *et al,* 2011; Giaginis *et al,* 2009). Several studies have demonstrated that AJA displays anti-inflammatory effects in animal arthritis models and inhibits the promoter activity of IL-8 (Ambrosio *et al,* 2007; Zurier *et al,* 2003). These effects in part may be PPARγ mediated (Liu *et al,* 2003). Furthermore, the synthetic cannabinoid AJA has been shown to increase the production of 15d-PGJ_2 a product of anandamide cleavage (Stebulis *et al,* 2008). 15d-PGJ_2 is a ligand of PPARγ and was shown to counteract IL-1β induced COX-2, and iNOS expression and also the production of NO and the decrease in proteoglycan in human chondrocytes (Boyault *et al,* 2001). 15d-PGJ_2 also inhibited the activation of NFκB and attenuated AP-1 binding to DNA in human chondrocytes (Boyault *et al,* 2001). Conversely, PPARγ agonist troglitazone, used in the treatment of type 2 diabetes, had no significant inhibitory effects on IL-1β-stimulated iNOS and COX-2 expression indicating the actions of 15d-PGJ_2 may be PPARγ independent (Boyault *et al,* 2001; Bianchi *et al,* 2005). Furthermore, 15d-PGJ_2 decreased IL-1β induced NO and MMP-13 production in chondrocytes and also inhibited IL-1β induced MMP-1 production in human synovial cells (Fahmi *et al,* 2001; Fahmi *et al,* 2002).

In animal models of arthritis non-cannabinoid PPARγ agonist piogitazone reduced the development and severity of cartilage lesions (Kobayashi *et al,* 2005; Boileau *et al,* 2007). Activation of PPARγ may therefore inhibit the actions

of IL-1β in matrix degradation by reducing pro-inflammatory mediators and matrix degrading enzymes. There is increasing evidence to show that these receptors are also involved in inflammation and cytokine modulation by inhibiting the expression of NF-κB, which induces pro-inflammatory responses (O'Sullivan 2007).

Activation of PPARα using a selective PPARα agonist Wy-14643, reduced IL-1β stimulated production of MMPs and the production of inflammatory mediators NO and PGE_2 in human OA cartilage explants, however there were no effects on collagen type II or aggrecan expression (Clockaerts *et al,* 2011) suggesting that these receptors could play a role in the inflammatory process of arthritic disease when activated by specific ligands. In another study activation of PPARα using a specific agonist increased the production of IL-1 Ra in chondrocytes indicating that activation of these receptors may protect chondrocytes against IL-1β induced responses (Francois *et al,* 2006). PPAR activation induced by cannabinoids may also have effects on inflammatory mediators involved in the pathogenesis of OA. An example of this is endogenous cannabinoid PEA, which mediates its anti-inflammatory effects by activating PPARα (Lo Verme *et al,* 2005). WIN-55 also binds to and increases the transcriptional activity of PPARα (Sun *et al,* 2006) and AEA binds to and activates both PPARα and PPARγ (Bouaboula *et al,* 2005; Sun *et al,* 2006; Sun and Bennett 2007).

There is little knowledge regarding the chondroprotective activities of PPARδ however, activation of PPARδ in rat synovial fibroblasts was shown to stimulate production of IL-1Ra, suggesting that PPARδ may have potential anti-arthritic properties (Moulin *et al,* 2005).

Endogenous cannabinoids AEA and 2-AG have been detected in the synovial fluid of OA and RA patients, however these endogenous cannabinoids were not detected in the synovial fluid of normal patients (Richardson *et al,* 2008) indicating upregulation of their expression in arthritis. Interestingly, PEA and OEA levels were decreased in the synovial fluid of patients with OA and RA compared to controls (Richardson *et al,* 2008). Furthermore, AEA has been shown to have anti-inflammatory effects via suppressing TNF induced NF-κB

activation by inhibiting IκB kinase independent of CB1 and CB2 receptors (Sancho *et al,* 2003). These studies suggest that the endogenous cannabinoid system may also be a target for anti-arthritic therapies.

1.16 Summary

A key pathological feature of OA is cartilage degradation. ECM breakdown in OA is primarily driven by the pro-inflammatory cytokine IL-1β that induces the upregulation of MMPs (Kapoor *et al,* 2011). In addition IL-1β inhibits anabolic activities of chondrocytes decreasing collagen type II and aggrecan (Goldring *et al,* 1994; Chadjichristos *et al,* 2003; Stove *et al,* 2000). Thus IL-1β significantly contributes to the dysregulation of ECM turnover during OA. Currently there are no therapies to prevent cartilage degradation in OA, ultimately leading to total joint replacements. Targeting inhibition of IL-1β signalling pathways inappropriately activated during OA is therefore a key therapeutic target.

Cannabinoids have been shown to reduce joint damage in animal models of arthritis and present therapeutic possibilities in the prevention of cartilage breakdown (Figure 1.11) (Malfait *et al,* 2000; Sumariwalla *et al,* 2004; Zurier *et al,* 1998). Furthermore, the activation of certain cannabinoid receptors namely PPARα and γ display anti-inflammatory properties in addition to inhibiting destructive pathways in OA (Fahmi *et al,* 2001; Boyault *et al,* 2001; Clockaerts *et al,* 2011). Improved understanding of how cannabinoids such as WIN-55 may act to prevent cartilage breakdown will identify potential therapeutic agents. In addition, identification of cannabinoid receptors within different grades and zones of OA cartilage will provide insight into the expression patterns of cannabinoid receptors during disease progression.

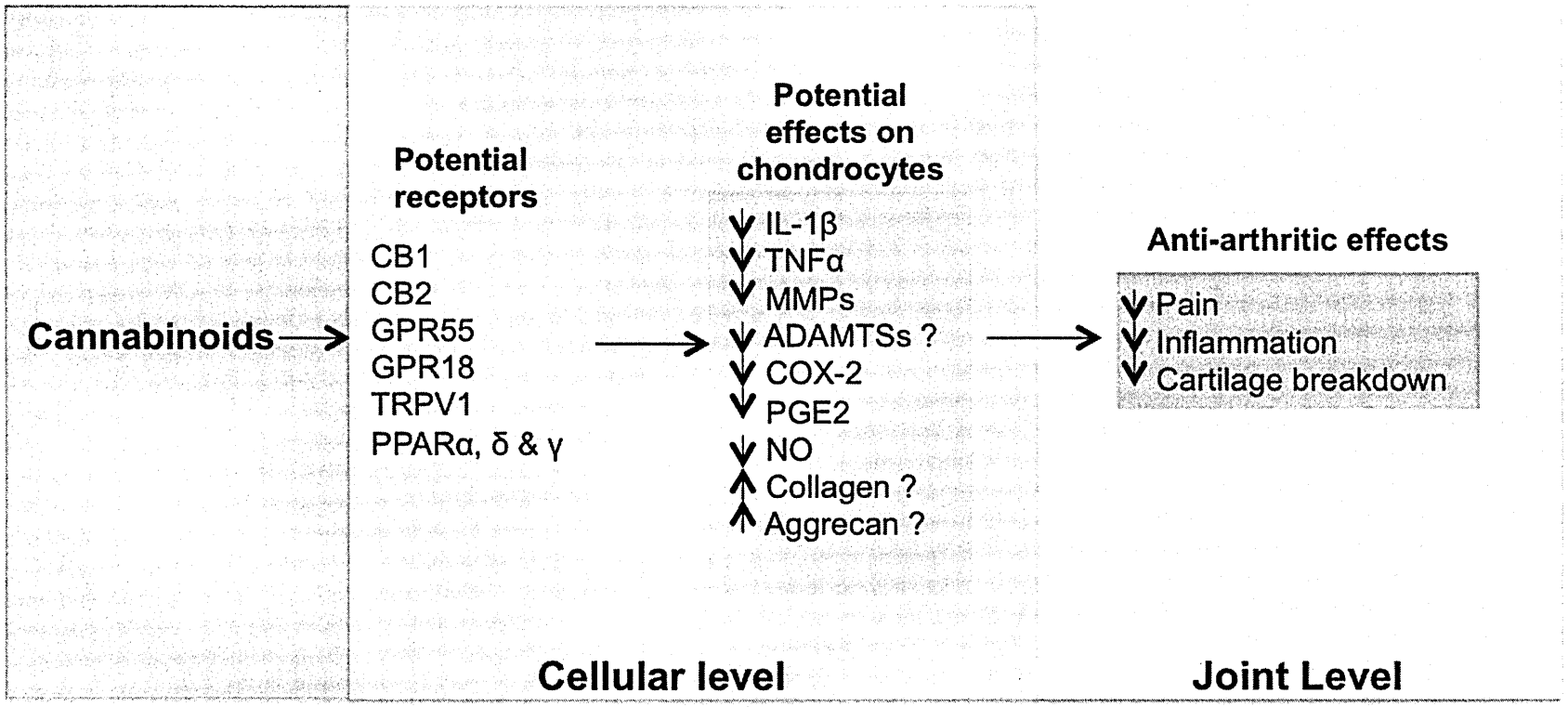

Figure 1.11 Potential targets of cannabinoid in relation to chondrocyte metabolism and arthritis. The diagram summaries the information taken from the literature in relation to cannabinoids and chondrocytes. ↓ indicates inhibitory effects; ↑ stimulatory effects; ? speculative effects (Dunn *et al* 2012).

1.17 Aims and Objectives

Global Aim/ Hypothesis: This thesis aimed to test the hypothesis i) that synthetic cannabinoid WIN-55 inhibits IL-1β induced catabolic pathways in OA chondrocytes; ii) that cannabinoid receptors are expressed in OA cartilage and bone cells and their expression is altered in different grades of disease and iii) activation of cannabinoid receptors with selective agonists decreases IL-1β induced catabolic pathways in OA chondrocytes.

Objectives:

- To determine the effects of synthetic cannabinoid WIN-55 on IL-1β induced expression of MMP-3 and -13 within different grades of OA chondrocytes.
- To determine the effects of synthetic cannabinoid WIN-55 on TIMP-1 and -2 expression within different grades of OA chondrocytes.
- To determine effects of WIN-55 on IL-1β signalling pathways
- To identify the expression and modulation of putative cannabinoid receptors within distinct histological grades of OA cartilage and cartilage zones and underlying bone.
- To determine which cannabinoid receptors mediate WIN-55 induced responses in OA chondrocytes based on selective agonist receptor activation.

2 The Effects of WIN-55 on IL-1β Induced Catabolic Pathways

2.1 Introduction

During OA there is a shift in the equilibrium between catabolic and anabolic activities in cartilage (Goldring and Marcu 2009). As a result the breakdown of collagen and proteoglycans may exceed the rate of synthesis of new matrix molecules resulting in cartilage degradation. Another contributing factor in cartilage breakdown in OA is an increase in inflammatory cytokines particularly IL-1β and TNFα produced by the articular chondrocytes or cells of the synovium (Goldring and Otero 2011). This results in an increase in MMPs particularly MMP-1, -3 and MMP-13, which are expressed in OA cartilage and synovial tissue, (Davidson *et al,* 2006; Bau *et al,* 2002; Hembry *et al,* 1995; Okada *et al,* 1992; Wolfe *et al,* 1993; Chubinskaya *et al,* 1999; Yoshihara *et al,* 2000; Koshy *et al,* 2002; Tetlow *et al,* 2001) without an increase in their inhibitors TIMPs (Dean *et al,* 1989; Martel-Pelletier *et al,* 1994). During OA an increase in MMPs over TIMPs leads to cleavage of ECM molecules principally aggrecan and collagen type II. MMP inhibition has been proposed as a possible mechanism to prevent breakdown of cartilage tissue in arthritis, provided the necessary functional specificity can be achieved (Murphy and Nagase 2008).

Cannabis-based medicine Sativex has been shown to have analgesic effects and to suppress disease activity in patients with RA (Blake *et al,* 2006). Cannabinoids also have anti-inflammatory effects and reduce joint damage in animal models of arthritis (Malfait *et al,* 2000; Sumariwalla *et al,* 2004; Zurier *et al,* 1998). *In vitro* studies have shown that cannabinoids reduce cytokine production from OA and RA fibroblasts and the release of matrix MMPs from fibroblast-like synovial cells (Johnson *et al,* 2007; Zurier *et al,* 2003; Selvi *et al,* 2008). They also have direct effects on cartilage ECM breakdown; reducing IL-1α induced proteoglycan and collagen degradation in bovine cartilage (Mbvundula *et al,* 2006). There is thus increasing evidence to suggest that cannabinoids have chondroprotective effects and may be of value in the treatment of arthritis (Dunn *et al,* 2012).

2.1.1 WIN-55

The synthetic cannabinoid WIN-55 is known to activate the classical cannabinoid receptors CB1 and CB2 in addition to the nuclear cannabinoid receptors including PPARα and γ (Pertwee *et al,* 2010; O'Sullivan 2007;Sun *et*

al, 2006). WIN-55 has been shown to display anti-inflammatory affects in human OA and RA synovial fibroblasts via reducing the secretion of IL-6 and IL-8 (Selvi *et al,* 2008). In bovine chondrocytes, WIN-55 was shown to prevent the IL-1α induced breakdown of proteoglycan and collagen (Mbvundula *et al,* 2006). Furthermore, in human astrocytes, WIN-55 has also been shown to modulate the activities of IL-1 via inhibition of NFκB (Curran *et al* 2005). Collectively, these findings suggest that WIN-55 displays both anti-inflammatory and anti-catabolic activities in a number of cell types.

2.1.2 Model Culture Systems

In monolayer, chondrocytes redifferentiate into fibroblast-like cells and change their matrix synthesis from collagen type II and aggrecan to collagen type I and X (Mayne *et al,* 1976; von der Mark *et al,* 1977; Benya *et al,* 1978). Phenotypic features of chondrocytes can be reversed and maintained when cultured in three-dimensional (3D) culture of alginate beads and cell pellets. Alginate is derived from brown seaweed as a polymer of β-D mannuronic acid and α-L-guluronic acid (Hauselmann *et al* 1996). Cells can be suspended in viscous alginate solution prior to polymerisation. In the presence of divalent ions including calcium, alginate forms a semi-solid gel-like matrix in which cells can be embedded to redifferentate them back to their native phenotype (Hauselmann *et al,* 1996). As the alginate is negatively charged it is thought to mimic the *in vivo* properties of the ECM of proteoglycans. Chondrocytes cultured in alginate beads produce a matrix of collagen type II and aggrecan (Hauselmann *et al,* 1996). The properties of alginate beads allows for live cells to be easily isolated from the matrix by the addition of chelating agents (De Ceuninck *et al,* 2004). Chondrocytes embedded and cultured in alginate beads are able to respond to stimulation from growth factors and cytokines allowing for the study of chondrocyte metabolism using various molecular techniques, including PCR (Beekman *et al,* 1998; Lemare *et al,* 1998; Loeser *et al,* 2003).

Cell pellets are another form of 3D culture system used to redifferentiate chondrocytes, which are formed by pelleting cells in conical tubes by centrifugation (Schulze-Tanzil *et al,* 2002). Cell pellets have been used for the redifferentation of primary chondrocytes to study the effects of growth factors and cytokines (Xu *et al,* 1996). Moreover histological analysis of cell pellets

show chondrocytes form lacunae and produce an extracellular matrix of collagen II and proteoglycans.(Dozin *et al,* 2002).

2.2 Aims and Objectives

Aim: This study aimed to investigate the effects of synthetic cannabinoid WIN-55 on MMP-3 and -13, TIMP-1 and -2 expression human OA chondrocytes in the presence of IL-1β.

- To determine the time-dependent effects of WIN-55 on the mRNA expression of MMPs and TIMPs.
- To determine the concentration-dependent effects of WIN-55 on the mRNA expression of MMPs and TIMPs.
- To investigate the effects of WIN-55 on the mRNA expression of MMPs and TIMPs in the presence of pro-inflammatory cytokine IL-1β in chondrocytes isolated from different macroscopic grades of OA cartilage both in monolayer and 3D culture.
- To determine the effects of WIN-55 on MMP-3 and -13 protein release into culture media following IL-1β stimulation in both monolayer and 3D culture.

2.3 Experimental Design

The effects of WIN-55 on matrix degrading enzymes MMP-3 and -13 and their inhibitors TIMP-1 and -2 in chondrocytes obtained from different grades of OA cartilage were investigated. Cartilage tissue was graded macroscopically 0-4 using the Outerbridge classification (Cameron *et al,* 2003). Chondrocytes were isolated from grade 0, 2 and 3 cartilage tissue as representative of non-degenerate, low degenerate and intermediate degenerate cartilage tissue. Cartilage from grade 4, severe degenerate tissue, was not used in the study as the cell yield obtained was not sufficient. Chondrocytes were cultured in monolayer and then transferred to alginate beads and cell pellets to redifferentiate chondrocytes back to their native phenotype for four weeks. Cells were stimulated with IL-1β to induce catabolic responses. Chondrocytes were also treated with WIN-55 with and without IL-1β and the gene expression of

MMP-3, -13, TIMP-1 and -2 investigated using real-time PCR. In order to determine the effects of WIN-55 on IL-1β stimulated MMP-3 and -13 protein production, enzyme-linked immunosorbent assay (ELISA) was used to measure the amount of active and pro-MMP-3 released from cells into the culture media from chondrocytes cultured in monolayer and alginate beads and the total and pro-MMP-13 released into the culture media from chondrocytes cultured in alginate beads following IL-1β and WIN-55 treatment. An MMP-3 enzyme activity assay was also used to measure MMP-3 activity in the conditioned culture media following IL-1β and WIN-55 treatment.

2.4 Methodology

2.4.1 Human OA Cartilage Samples

Primary Human chondrocytes were obtained from the articular cartilage removed from patients with symptomatic OA at the time of total knee replacement (Ethical approval gained through Sheffield Musculoskeletal Biobank). All patients provided written, informed consent prior to participation and tissue samples were supplied by the Sheffield Biorepository. Cartilage blocks were taken from each anatomic compartment within the knee (n=5-7 per patient) (medial and lateral tibio-femoral and patello-femoral compartments) (Appendix 1) and transported in Dulbecco's modified Eagle's medium (DMEM)/F-12 (1:1) (Invitrogen) supplemented with 2mM glutamine (Invitrogen), 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen), 2.5 µg/ml amphotericin B (Sigma-Aldrich) and 50 µg/ml ascorbic acid (Sigma-Aldrich) (serum free media).

2.4.2 Macroscopic Grading of Cartilage Tissue

Cartilage tissue was macroscopically graded 0-4 using the Outerbridge Classification at time of surgery by Prof J.M.Wilkinson, orthopaedic surgeon prior to isolation of chondrocytes (Cameron *et al,* 2003). Cartilage was classified into macroscopic grades (Table 2.1).

Macroscopic Grade	Cartilage Features
Grade 0	Normal
Grade I	Cartilage with softening and swelling
Grade II	A partial thickness defect with fissures on the surface that do not reach subchondral bone or exceed 1.5 cm in diameter
Grade III	Fissures that reach the level of subchondral bone in an area with a diameter of more than 1.5 cm
Grade IV	Exposed subchondral bone

Table 2.1 Outerbridge Classification of human cartilage tissue at time of total knee replacement

2.4.3 Isolation of OA Chondrocytes

Cartilage was removed from the bone, finely dissected and washed twice in 1xphosphate buffered saline (PBS) (Invitrogen). Cartilage was digested in 0.25% trypsin (Sigma-Aldrich) at 37°C for 30 minutes followed by digestion in 3 mg/ml collagenase type I (Sigma-Aldrich) in DMEM/F-12 (1:1) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen), 2mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B and 50 µg/ml ascorbic acid (complete media), at 37°C for 16 hours. The resulting cell suspension was passed through a 70 µm cell strainer (Fisher Scientific) and centrifuged at 400g for 10 minutes; the cell pellet was then washed twice in serum free media, followed by centrifugation at 400g for 10 minutes. The cell pellet was resuspended in complete media. Cells were counted and the viability checked using trypan blue exclusion on the Countess cell counter (Invitrogen) (approximately $5x10^5$ cells/ml of directly extracted cells). Cells were seeded in a T75 flask (Nunc) and maintained in DMEM/F12 complete media in a humidified atmosphere of 5% CO_2 and the culture media changed every other day.

2.4.4 OA Patient Samples

Chondrocytes cultures were derived from OA patient samples; HC1(1), HC4(2) HC5(2), HC5(4), HC6(1), HC7(1), HC11(3), HC15(4), HC16(4), HC17(4), HC20(1) and HC23(4) (Table 2.1). HC is human cartilage with sample number and the anatomic compartment code in brackets. Full patient sample information can be found in Appendix 1.

2.4.5 Monolayer Culture: Cell Passaging

Cells were cultured in monolayer until 80% confluent before passaging. At passage 0 chondrocytes display a rounded morphology, by passage 1 and 2 the chondrocytes are flatter with a fibroblast-like elongated morphology (Figure 2.1). Culture media was removed and cells were washed twice in 1X PBS and incubated with trypsin/ethylenediamine tetraacetic acid (trypsin-EDTA) (Invitrogen) at 37°C for 5 minutes to detach cells. The trypsin–EDTA was inactivated by the addition of complete culture media and centrifuged at 400g for 10 minutes and resuspended in complete culture media. Cells were split at a ratio of 1:3 into T75 flasks and maintained in culture until passage 2 in complete culture media in a humidified atmosphere of 5% CO_2 and the culture media changed every other day prior to IL-1β and WIN-55 treatment as outlined in sections 2.4.6 and 2.4.7.

2.4.6 IL-1β and WIN-55 Treatment of OA Chondrocytes Cultured in Monolayer for Investigation of Time and Concentration Dependent Effects

Chondrocytes were cultured in monolayer until 80% confluent at passage 2. Following trypsinisation as outlined in section 2.4.5, cells were centrifuged at 400g for 10 minutes and resuspended in complete media. Cells were counted using trypan blue exclusion on the Countess cell counter. Cells were seeded in 6 well culture plates at a cell density of $5x10^5$ per well. Cells were allowed to adhere overnight at 37°C in a humidified atmosphere of 5% CO_2. Complete media was removed and cells washed twice with 1xPBS. Media containing 500 µg/ml bovine serum albumin (BSA) (Sigma-Aldrich), 2mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B and 50 µg/ml ascorbic acid (Serum free media+BSA) supplemented with 10 ng/ml IL-1β (Peprotech) with and without 1 µM, 2.5 µM, 5 µM, 7.5 µM and 10 µM WIN-55 (Sigma-Aldrich) was added to the cells and they were incubated for 48 hours at 37°C. Chondrocytes in monolayer were also treated alone with 10 µM WIN-55 for 3, 6 24 and 48 hours at 37°C. Dimethyl sulfoxide (DMSO; Sigma-Aldrich) (0.1%) was used as vehicle control at the same concentration present in 10 µM WIN-55 treatment. Each treatment was performed in triplicate in grade 0 chondrocytes (Table 2.2).

2.4.7 IL-1β and WIN-55 Treatment of OA Chondrocytes Cultured in Monolayer Isolated from Different Macroscopic Grades of Cartilage

Chondrocytes were seeded in 6 well culture plates at a cell density of $5x10^5$ per well as described in section 2.4.6. Serum free media + BSA supplemented with 10 ng/ml IL-1β with and without 10 μM WIN-55 was added per well for 48 hours. DMSO (0.1%) was used as vehicle control at the same concentration present in 10 μM WIN-55 treatment. Each treatment was performed in triplicate on grade 0, 2 and 3 isolated chondrocytes (Table 2.2). Following treatment, conditioned culture media was stored at -20° for MMP-3 and -13 cell based ELISA and MMP-3 enzyme activity assay analysis (sections 2.4.22 and 2.4.24).

Analysis Performed	Monolayer Culture			Alginate Beads			Cell Pellets		
	Grade 0	**Grade 2**	**Grade 3**	**Grade 0**	**Grade 2**	**Grade 3**	**Grade 0**	**Grade 2**	**Grade 3**
MMP-3 mRNA Expression	HC5(2) HC6(1) HC7(1) HC20(1)*	HC1(1) HC23(4) HC17(1)	HC11(3) HC15(4) HC16(4)	HC5(2) HC6(1) HC7(1)	HC1(1) HC5(4) HC17(1)	HC11(3) HC15(4) HC16(4)	HC5(2) HC6(1) HC7(1)	HC1(1) HC5(4) HC17(1)	HC11(3) HC15(4) HC16(4)
MMP-13 mRNA Expression	HC5(2) HC6(1) HC7(1) HC20(1)*	HC1(1) HC23(4) HC17(1)	HC11(3) HC15(4) HC16(4)	HC5(2) HC6(1) HC7(1)	HC1(1) HC5(4) HC17(1)	HC11(3) HC15(4) HC16(4)	HC5(2) HC6(1) HC7(1)	HC1(1) HC5(4) HC17(1)	HC11(3) HC15(4) HC16(4)
TIMP-1 mRNA Expression	HC5(2) HC6(1) HC7(1) HC20(1)*	HC1(1) HC23(4) HC17(1)	HC11(3) HC15(4) HC16(4)	HC5(2) HC6(1) HC7(1)	HC1(1) HC5(4) HC17(1)	HC11(3) HC15(4) HC16(4)			
TIMP-2 mRNA Expression	HC5(2) HC6(1) HC7(1) HC20(1)*	HC1(1) HC23(4) HC17(1)	HC11(3) HC15(4) HC16(4)	HC5(2) HC6(1) HC7(1)	HC1(1) HC5(4) HC17(1)	HC11(3) HC15(4) HC16(4)			
MMP-3 Protein Release	Not Investigated	HC5(4)	Not Investigated	Not Investigated		HC11(3) HC15(4) HC16(4)	Not investigated		
MMP-13 Protein Release	Not Investigated			Not Investigated		HC11(3) HC15(4) HC16(4)			

MMP-3 Enzyme Activity	Not Investigated	HC5(4)	Not Investigated	Not Investigated
MTS Cell Viability Assay	Not Investigated		HC23(4) HC4(2)	Not Investigated

Table 2.2 The patient samples used for each analysis performed in each of the culture systems in chondrocytes obtained from different macroscopic grades of OA cartilage. Full details of samples used in these investigations can be found in Appendix 1. *Indicates the patient sample used for investigation of time and dose-dependent effects of WIN-55. HC is human cartilage with sample number and the anatomic compartment code shown in brackets (Appendix 1).

2.4.8 Alginate Bead Culture

Cells cultured in monolayer were harvested at passage 2 and transferred into alginate beads. Chondrocytes suspended in alginate beads regain their rounded morphology (Figure 2.1). Following trypsinisation as outlined in section 2.3.5. cell suspensions were centrifuged at 400g for 10 minutes and the resulting cell pellet was resuspended in 10 ml of complete culture media and the cell number determined using trypan blue exclusion and the Countess cell counter. Following further pelleting the chondrocytes were resuspended in 1.2% medium viscosity sodium alginate (Sigma-Aldrich) in 0.15M sodium chloride (NaCl) at a cell density of $2x10^6$ per ml of alginate. The resulting cell suspension was passed through a 19 gauge needle into a 12 well plate containing 2 ml of 200 mM calcium chloride ($CaCl_2$) solution, where each drop was instantly polymerised forming semisolid microspheric beads. The beads were polymerised at 37°C for 10 minutes and then washed twice with 0.15M NaCl and twice in serum free media. Alginate beads were cultured in complete media in a humidified atmosphere of 5% CO_2 at 37°C and the media changed every other day (Figure 2.1). Alginate beads were redifferentiated in culture for 4 weeks in 2 ml complete media prior to IL-1β and WIN-55 treatment (section 2.4.9).

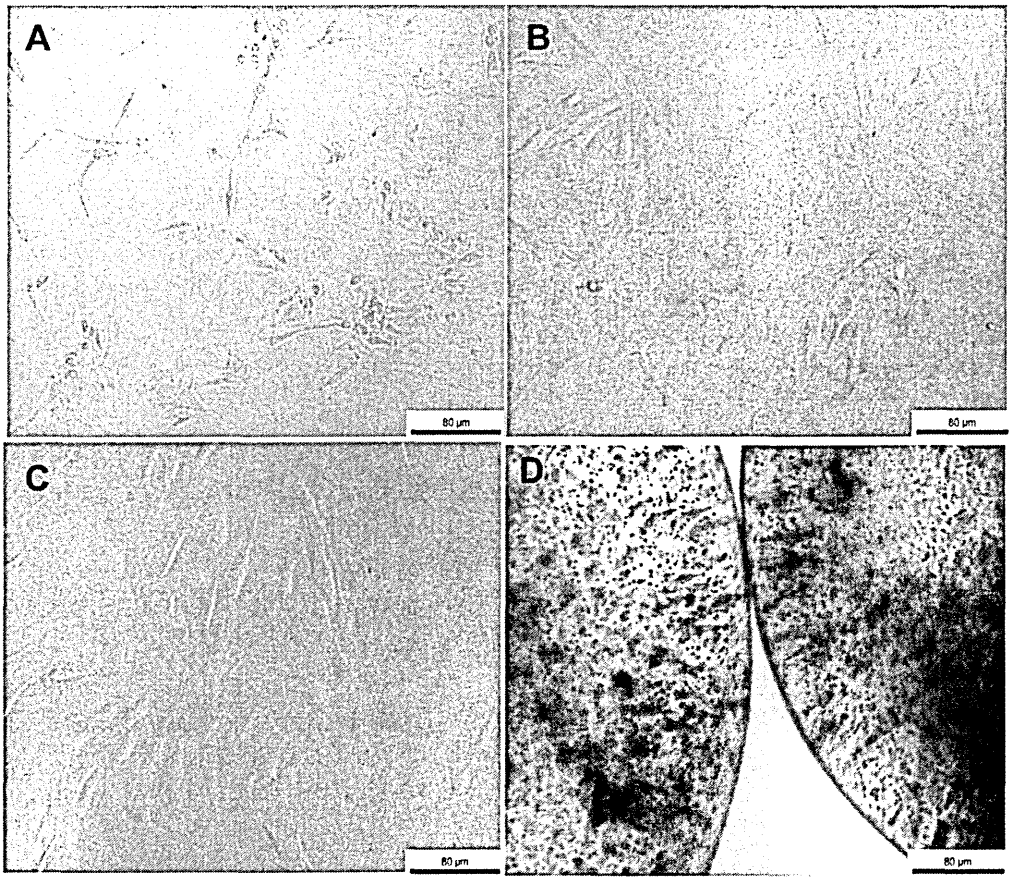

Figure 2.1 Primary human chondrocytes cultures. (A) Monolayer culture Passage 0 (P0) (B) Passage 1 (P1) (C) Passage 2 (P2) and (D) alginate bead culture. At P0 chondrocytes display a rounded morphology, by P1 and P2 the chondrocytes are flatter with a fibroblast-like elongated morphology. Following suspension in alginate beads chondrocytes regain their rounded morphology. Cultures obtained from patient sample HC20(1).

2.4.9 IL-1β and WIN-55 Treatment of Chondrocytes Cultured in Alginate Beads.

2 alginate beads were placed per well in 12 well plates (Nunc). Beads were washed with serum free media and 2 ml serum free+BSA media supplemented with 10 ng/ml IL-1β with or without 10 μM WIN-55 added per well and incubated for 48 hours. DMSO (0.1%) was used as a vehicle control at the same concentration present in 10 μM WIN-55 treatment. Each treatment was performed in triplicate on grade 0, 2 and 3 isolated chondrocytes (Table 2.2). Following treatment, conditioned culture media was stored at -20°C for cell based ELISA analysis (section 2.4.22).

2.4.10 Cell Pellet Culture

Cells cultured in monolayer were harvested at passage 2 and transferred to cell pellets. Following trypsinisation as outlined in section 2.3.5 cells were centrifuged at 400g for 10 minutes and the resulting cell pellet was resuspended in 10 ml of complete culture media and the cell number counted using trypan blue exclusion on the Countess cell counter. Chondrocytes were centrifuged at 400g for 10 minutes at a cell density of $2x10^5$ cells/pellet in 2 ml of complete culture media. The resulting cell pellets were cultured in 15 ml falcon tubes (Fisher Scientific) in a humidified atmosphere of 5% CO_2 at 37°C, and the media changed every other day. Cells were redifferentiated in cell pellets for four weeks prior to IL-1β and WIN-55 treatment (section 2.4.11).

2.4.11 IL-1β and WIN-55 Treatment of Chondrocytes Cultured in Cell Pellets.

Cell pellets were washed with 1xPBS and 1 cell pellet was used per treatment. 2 ml serum free media+BSA supplemented with 10 ng/ml IL-1β with or without 10 μM WIN-55 was added per 15 ml falcon tube and incubated for 48 hours. DMSO (0.1%) was used as vehicle control at the same concentration present in 10 μM WIN-55 treatment. Each treatment was performed in triplicate on grade 0, 2 and 3 isolated chondrocytes (Table 2.2).

2.4.12 Cytotoxicity Studies MTS Assay

The CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (MTS) (Promega) was used to determine the effects of 10μM WIN-55 on the cell viability of human chondrocytes. At passage 2 cells, trypsinised as outlined in

section 2.4.5, were centrifuged at 400g for 10 minutes and the resulting cell pellet resuspended in 10 ml of complete culture media and the cell number counted using trypan blue exclusion on the Countess cell counter (Invitrogen). Cells were seeded at a cell density of $1x10^5$ cells per well in a 96 well culture plate and cells were allowed to adhere overnight in a humidified atmosphere of 5% CO_2 at 37°C. Serum free media+BSA (200 µl) supplemented with 10 ng/ml IL-1β with and without 10 µM WIN-55 was added to each well and incubated for 48 hours at 37°C. DMSO (0.1%) was used as vehicle control at the same concentration present in 10 µM WIN-55 treatment. Each treatment was performed in triplicate using two patient samples (Table 2.2). Following treatments 40 µl of MTS solution was added to each well and incubated at 37°C for 4 hours. The absorbance was read at 490 nm using the Wallac Victor 1820 plate reader. Average absorbances were taken for each reading and the results expressed as percentage cell viability compared to control cells.

2.4.13 RNA Extraction from Cells Cultured in Monolayer

Culture media was removed from cells and 1 ml of TRIzol (Ambion) reagent was added to each well and incubated at room temperature for 5 minutes. The resulting TRIzol/cell suspensions were transferred to 1.5 ml Eppendorfs, 200 µl of chloroform added to each sample and then vortexed for 15 seconds and incubated at room temperature for 3 minutes. The samples were centrifuged at 12,000g for 15 minutes at 4°C and the aqueous phase transferred to a fresh 1.5 ml Eppendorf to which 500 µl of isopropanol was added and the samples incubated at room temperature for 10 minutes and then incubated at -80°C for at least 1 hour. The resulting RNA precipitate was centrifuged at 12,000g for 30 minutes at 4°C. The RNA pellet was washed with 80% ethanol and centrifuged at 7,500g for 15 minutes at 4°C and the RNA pellet air dried on ice for 30 minutes and resuspended in 14 µl of sterile deionised water (sdH_2O). RNA purity was determined using a Nanodrop (Thermo Scientific).

2.4.14 RNA Extraction from Alginate Beads using TRIzol and Qiagen RNA Clean-up Column

Following treatments RNA extraction from alginate beads was performed using TRIzol reagent for measurement of MMP-3, -13, TIMP-1 and -2 mRNA. Alginate

beads were removed from the cell culture media and transferred to 1.5 ml Eppendorfs and incubated in 1 ml dissolving buffer (55 mM sodium citrate, 30mM Ethylenediaminetetraacetic acid (EDTA), 0.15M NaCl, pH 6) at 37°C for 20 minutes. The resulting cell suspension was centrifuged at 600g for 15 minutes and the supernatant discarded. The cell pellet was resuspended in 0.06% collagenase type I v/v in complete culture media and incubated at 37°C for 20 minutes and centrifuged at 600g for 15 minutes forming at cell pellet. The resulting cell pellet was incubated with 1 ml of TRIzol reagent for 5 minutes at room temperature, 200 µl of chloroform was added to each sample and vortexed for 15 seconds. The samples were incubated at room temperature for 3 minutes and then centrifuged at 12,000g for 15 minutes. The aqueous phase was transferred to a new tube and 500 µl of isopropanol added and incubated at room temperature for 10 minutes. The samples were centrifuged at 12,000g for 30 minutes and the supernatant removed. The resulting RNA was resuspended in 100 µl of sterile deionised water and the RNA was purified using RNeasy clean up columns (Qiagen) according to the manufacturer's instructions. RNA purity was determined using a NanoDrop.

2.4.15 RNA Extraction from Cells Cultured in Pellets using TRIzol.

RNA extraction from cells cultured in cell pellets was performed using TRIzol reagent for the measurement of MMP-3 and -13 mRNA. Cell pellets were removed from culture media and transferred to 1.5 ml Eppendorfs. Cell pellets were incubated in 0.06% collagenase type I (Sigma-Aldrich) at 37°C for 15 minutes and centrifuged at 600g for 15 minutes, forming a cell pellet. The resulting cell pellets were incubated with 1 ml of TRIzol at room temperature for 5 minutes and RNA extracted as outlined in section 2.4.13.

2.4.16 Reverse Transcription

RNA was denatured at 60°C for 5 minutes. Reverse transcription (RT) of RNA to cDNA was performed using Bioscript reverse transcriptase (Bioline) prior to real-time polymerase chain reaction (PCR). RT mastermix was added to each sample (Table 2.3). Reverse transcription was performed at 42°C for 1 hour followed by 10 minutes at 80°C. cDNA was stored at -20°C for use in real-time PCR.

Reagent for 1 reaction	µl
Reverse transcriptase enzyme	0.5
dNTPs (40 nM)	1
Random Hexamers (50 µM)	1.5
Sterile deionised water	28

Table 2.3 Reverse transcription mastermix

2.4.17 The Principle of Taqman Real-Time PCR

Real-time PCR allows the quantitation of gene expression. Taqman real-time PCR utilises fluorescent probes that bind to specific sequences on target genes. The probe has a fluorescent reporter dye attached to it 5' end and a quencher dye at its 3' end (Figure 2.2). During extension of the primers the 5' exonuclease activity of Taq DNA polymerase cleaves the probe releasing the fluorescent reporter dye from the quencher dye, thus resulting in an increase in fluorescence, which is detected by the StepOnePlus ABI PCR machine. The baseline defined as the reporter fluorescent signal, which is below the limits of detection of the instrument. The baseline is subtracted from the fluorescent data and the measured fluorescence is expressed as an amplification plot (Figure 2.3). The C_T value is defined as the PCR cycle number at which the reporter fluorescent is greater than that of the fixed threshold for each gene which is set in the exponential region of the amplification plot (Figure 2.3). The presence of more cDNA template at the start of the reaction results in a lower C_T value as there are less cycles required for the fluorescent signal to reach the threshold.

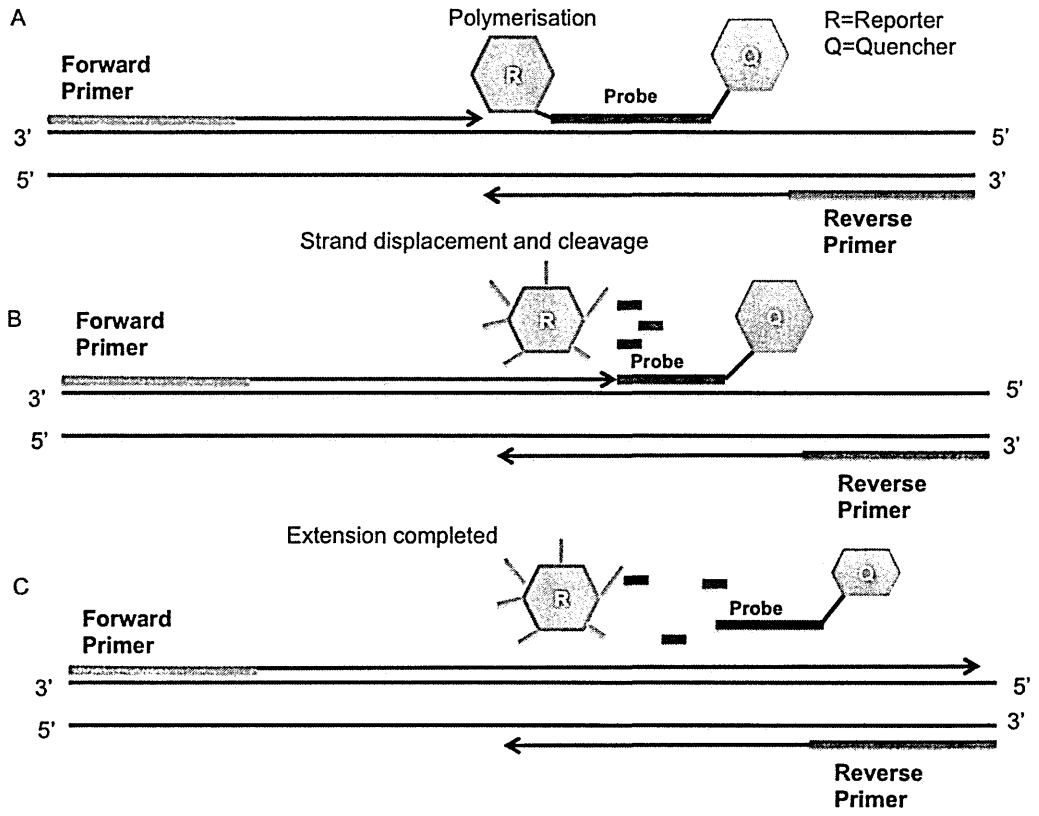

Figure 2.2 Real-time PCR principle. (A) The primers and probes containing a reporter and a quencher anneal to the complementary sequence of the target gene. Whilst in close proximity, the quencher, quenches the reporters fluorescence. **(B)** A Taq polymerase extends the forward primer sequence, as it reaches the annealed probe sequence it degrades the probe therefore releasing the reporter dye from the quencher and emitting a fluorescent signal which is directly detected by the Step One Plus ABI real-time PCR machine. **(C)** As the reaction continues Taq Polymerase further degrades the probe and completes the extension of the primers. This process is repeated for each PCR cycle.

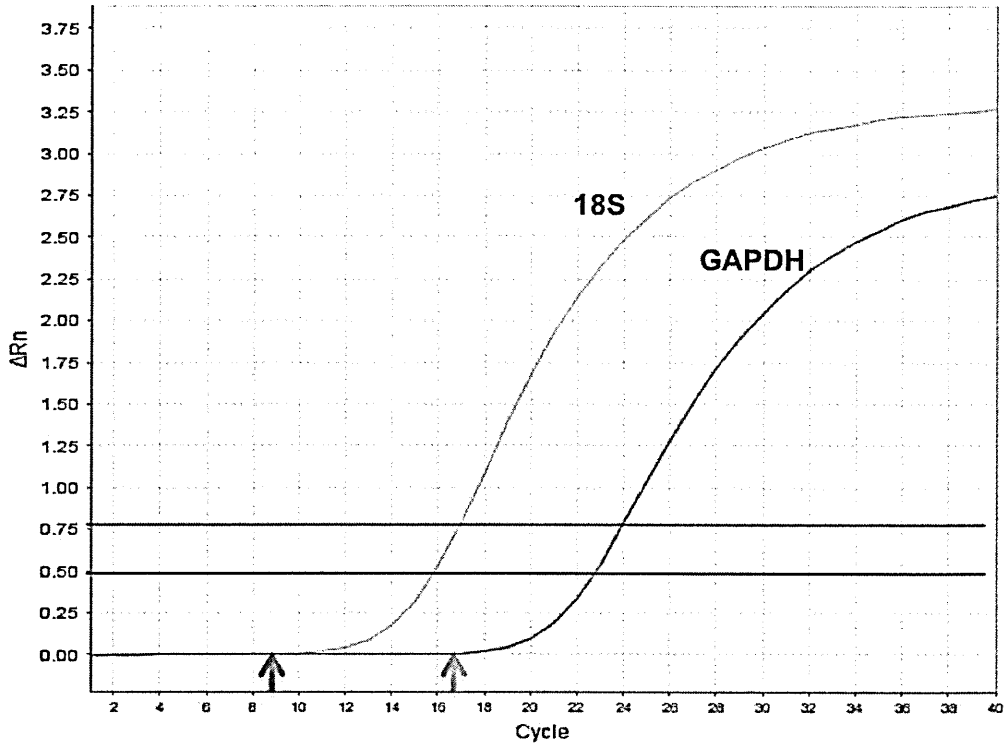

Figure 2.3 PCR amplification plot showing the threshold and baseline settings. Following amplification the base line was set for each target gene at two cycles prior to the first amplification curve. Thresholds were set in the exponential phase of the curve for each target gene. The red arrow indicates baseline setting of 9 for 18S gene amplification and a threshold of 0.75 within the exponential phase of the curve. The blue arrow indicates the baseline setting of 17 for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene amplification and a threshold of 0.5 within the exponential phase of the curve.

2.4.18 Real-time PCR

Taqman PCR was performed on cDNA samples from monolayer, alginate beads and cell pellets using pre-designed Taqman Gene Expression Assays (Table 2.4; Life Technologies). Gene expression was normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and eukaryotic 18S rRNA (18S) housekeeping genes (Table 2.4; Life Technologies). cDNA was diluted 1:10 prior to real-time PCR analysis and 2 µl was used in duplicate in 96 well Fast Optical Density PCR plates (Life Technologies). Real-time PCR master mix was prepared using 8 µl per reaction/well (Table 2.5).

Separate mastermix was loaded for each target gene and housekeeping gene. PCR plates were sealed with Fast Optical Adhesive Covers (Life Technologies) and PCR was run on an Applied Biosystems StepOnePlus Real-Time PCR

machine for 40 cycles of denaturation at 95°C for 1 second followed by annealing and extension at 60°C for 20 seconds.

Taqman Gene Expression Assay	Assay ID
GAPDH	Hs9999905_m1
18S	Hs99999901_s1
MMP-3	Hs00968305_m1
MMP-13	Hs00233992_m1
TIMP-1	Hs00171558_m1
TIMP-2	Hs00234278_m1

Table 2.4 Taqman gene expression assay IDs

For 1 Reaction	µl
Taqman Gene Expression Assay	0.5
Taqman FAST Mastermix	5.0
Sterile deionsed H_2O	2.5
Total	8.0

Table 2.5 Taqman FAST mastermix

2.4.19 Analysis of Real-Time PCR

Following amplification, the baseline was set up to 2 cycles prior to the first amplification for each target and housekeeping gene (Figure 2.3). The threshold for each was set within the exponential phase of the PCR amplification curve (Figure 2.3). The same threshold was used for each individual target. The C_T values were exported to excel and the data analysed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) to determine target mRNA expression relative to the internal housekeeping gene reference.

2.4.20 $2^{-\Delta\Delta CT}$ Analysis

The $2^{-\Delta\Delta CT}$ is a relative quantification method that relates the PCR cycle number of the target gene in a treatment group to that of another treatment group (or untreated control) (Lival&Schmittgen 2001).

1. C_T value duplicates were averaged for all target genes and housekeeping genes (GAPDH & 18S).
2. The housekeeping genes C_T values (GAPDH and 18S) were averaged.
3. ΔC_T values were calculated by subtracting the averaged housekeeping gene C_T value from the target gene C_T value.

   $\Delta C_T = C_T$ (target gene) $- C_T$ (mean reference gene)

4. Averages and the standard error of each data set were calculated.
5. $\Delta\Delta C_T$ values were calculated

   $\Delta\Delta C_T$ = mean ΔC_T (treatment group) – mean ΔC_T (untreated control)

6. Target gene expression relative to the internal reference gene and control expression was calculated

   Relative gene expression = $2^{-\Delta\Delta CT}$

7. Error bars representing standard error of ΔC_T values were calculated

   Positive Error = $2(^{-\Delta\Delta CT} + ^{\Delta CT\ SE}) - 2^{-\Delta\Delta CT}$
   Negative Error = $2^{-\Delta\Delta CT} - 2(^{-\Delta\Delta CT - \Delta CT\ SE})$

2.4.21 Taqman Primer/Probes Design and Optimisation

For each set of Taqman assays the efficiency of the amplification was determined. Following serial dilutions of cDNA template (1:10, 1:100, 1:1000, 1:10,000), cDNA was amplified in triplicate. The $\log_{10}$ of the cDNA concentration was applied and the amplification efficiency of the slope was calculated using

the line of best fit. A slope of 1 corresponded to one additional PCR cycle required to reach threshold, demonstrating 100% efficiency. Efficiency graphs with slopes between 0.9 and 1.1 with a correlation co-efficient (R^2) of at least 0.9 were accepted as efficient. All primer efficiencies are shown in Appendix 2.

2.4.22 MMP-3 and -13 Enzyme Linked Immunosorbent Assay (ELISA)

Pro-MMP-3 and total and pro-MMP-13 release into cell culture media from grade 3 chondrocytes cultured in monolayer and alginate beads following treatment with WIN-55 and IL-1β for 48 hours was determined using R&D Systems Quantikine ELISA kit (Table 2.2) (Figure 2.4). Briefly, samples were centrifuged at 400g for 10 minutes to remove particulates and were diluted accordingly with calibrator diluent RD5-10 (Table 2.6). MMP-3 standards of 10, 5, 2.5, 1.25, 0.625, 0.312 and 0.156 ng/ml from a stock solution of 100 ng/ml and MMP-13 standards of 5000, 2500, 1250, 625, 313, 156 and 78 pg/ml from a stock solution of 50,000 pg/ml were produced. Calibrator diluent served as the zero control standard (0 ng/ml). To the pre-coated wells 100 µl of assay diluent RD1-52 was added to each well and 100 µl of standard, control or sample was then added and incubated at room temperature for 2 hours on an orbital microplate shaker at 500 rpm. Each well was washed four times with 400 µl of wash buffer and 200 µl of MMP-3 or MMP-13 conjugate was added to each well and incubated at room temperature for 2 hours on an orbital microplate shaker at 500 rpm. Each well was washed four times in 1xwash buffer and 200 µl of substrate solution was added to each well and incubated at room temperature for 30 minutes protected from light. 50 µl of stop solution was then added to each well and the absorbance read at 450 nm and at 570 nm using the Wallac Victor 1820 plate reader (PerkinElmer).

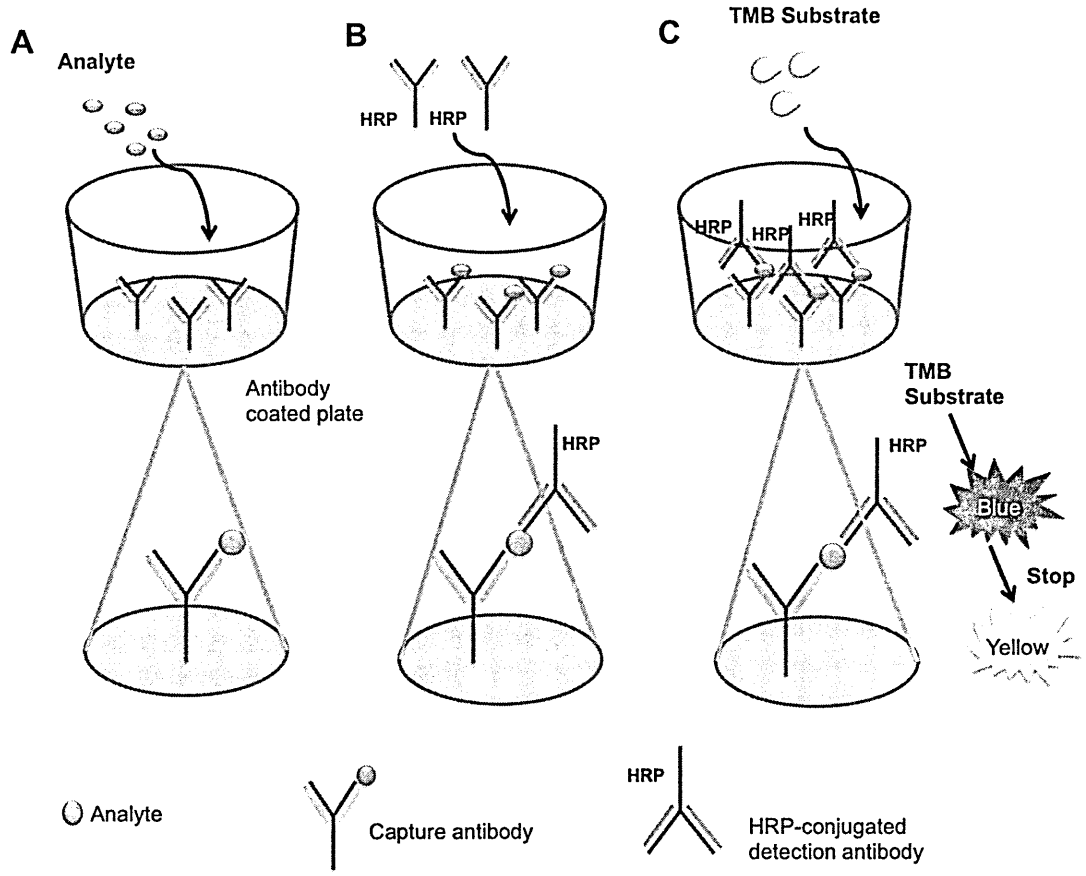

Figure 2.4 Principle of R&D Systems MMP-3 and -13 ELISA. (A) Samples are added to the microplate pre-coated with capture antibody, any analyte present is bound to the immobilised capture antibody. **(B)** A second horse radish peroxidase (HRP)-conjugated antibody is added and binds to the captured analyte. **(C)** Tetramethylbenzidine (TMB) substrate is added to each well and a blue colour develops, colour development is stopped and the absorbance is read at 450 nm.

Culture media sample	Dilution Factors		
	Monolayer MMP-3	Alginate MMP-3	Alginate MMP-13
Control	1:50	Not tested	Not tested
DMSO (0.1%)	1:50	1:50	Neat
IL-1β (10 ng/ml)+DMSO (0.1%)	1:1500	1:200	Neat
WIN-55 (10 μM)+ IL-1β (10 ng/ml)	1:10	1:1	Neat
WIN-55 (10 μM)	1:2	1:1	Neat

Table 2.6 MMP-3 and MMP-13 sample dilutions for ELISA analysis

2.4.23 MMP-3 and -13 ELISA analysis

The readings at 570 nm were subtracted from the readings of 450 nm to correct for optical imperfections in the plate. Each absorbance reading was averaged and the average zero standard optical density subtracted. A log/log standard curve was produced and a best fit line produced and the concentration of MMP-3 or MMP-13 in each sample determined. Each sample concentration was multiplied by the dilution factor for the individual samples and further normalised to the untreated control or DMSO control. For alginate beads, data was expressed as amount of protein production per alginate bead.

2.4.24 MMP-3 Activity Assay

The activity of MMP-3 released into the culture media from grade 2 isolated chondrocytes (Table 2.2) was measured using the BioVision MMP-3 Activity Assay Kit (Cambridge Bioscience, Cambridge, UK). During MMP-3 activity assay, MMP-3 hydrolyses a specific fluorescence resonance energy transfer (FRET) substrate to release the quenched fluorescent group methyl cumaryl amide (Mca), which can be detected fluorometrically. Mca standards were made of 0.5, 0.4, 0.3, 0.2 and 0.1 nM made from a 1 nM MMP-3 Mca stock. MMP-3 assay buffer served as the 0 nM standard. The standards were read

fluorometrically with the excitation at 325 nm and emission at 393 nm using the Tecan Infinite 200 Pro. Samples were centrifuged at 400g for 10 minutes to remove particulates and 50 µl of each sample added to a 96 well plate in duplicate. The provided MMP-3 served as a positive control and 10 µl was added to each well in duplicate and the final volume adjusted to 50 µl with MMP-3 assay buffer. Reaction mix was added to each well containing 2 µl of MMP-3 substrate and 48 µl of MMP-3 assay buffer. The samples and positive control were read fluorometrically with the excitation at 325 nm and emission at 393 nm at 4 minutes and the reaction was read again following incubation at room temperature for 1, 15, 20, 30, 40, 50, 60, 120, 180, 240, 300 and 360 minutes protected from light using the Tecan Infinite 200 Pro.

2.4.25 MMP-3 Activity Assay Analysis

The optimal incubation time was determined and fluorescence, measured in relative fluorescene units (RFU), was generated by the hydrolysis of the FRET substrate to release the quenched Mca fluorescent group to produce ΔRFU = R_2-R_1. The 0 standard was subtracted from the standard readings to produce the standard curve. The ΔRFU was applied to the standard to curve to get B nM of Mca (amount of unquenched Mca generated between T_1 and T_2) (Figure 2.5).

$$\text{MMP-3 Activity} = = \frac{B}{(T2-T1)x\,V} \text{x sample dilution factor=nM/min/ml = mU/ml}$$

B is nM Mca determined from the MMP Mca standard curve.

T_1 is the time of the first reading (R_1) (in min).

T_2 is the time of the second reading (R_2) (in min).

V is the pre-treated sample volume added to the reaction well (in ml).

2.5 Statistical Analysis

Data was shown to be non-parametric via a Shapiro–Wilk test hence statistical testing using Kruskal-Wallis multiple comparisons test was used to determine significance between DMSO vehicle control samples and IL-1β with and without WIN-55 treatment. The Conover-Inman post-hoc analysis was used to test when a significant difference was observed between different treatment groups. Statistical analysis was performed using StatsDirect software (StatsDirect Ltd).

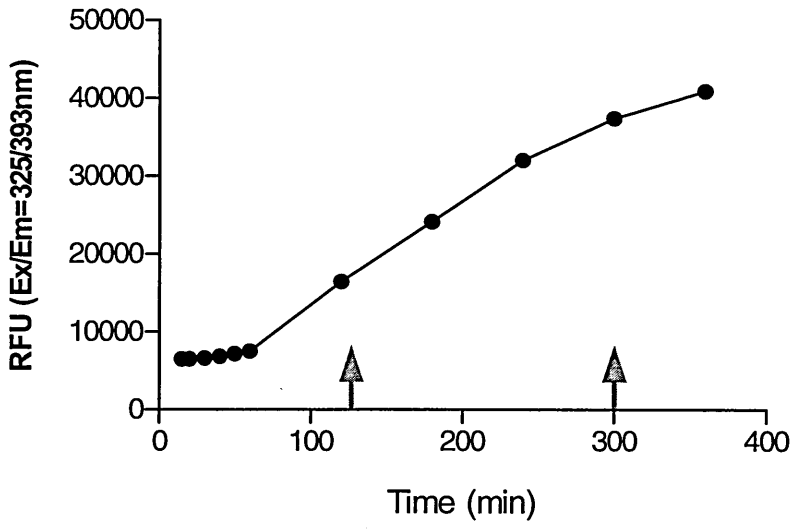

Figure 2.5 MMP-3 positive control activity overtime. The exponential phase of the reaction for the positive control is between 120 and 300 minutes as indicted by the red arrows, therefore these time points were used in the analysis of MMP-3 enzyme activity as outlined in section 2.4.25

2.6 Results

2.6.1 The Effects of WIN-55 on Chondrocyte Viability

WIN-55 treatment in combination with IL-1β for 48 hours reduced cell viability by 4% and WIN-55 treatment alone reduced cell viability by 7% compared to untreated control (Figure 2.6), however this was not significant ($p>0.05$), indicating that the concentration of WIN-55 used in this study did not significantly affect chondrocyte viability.

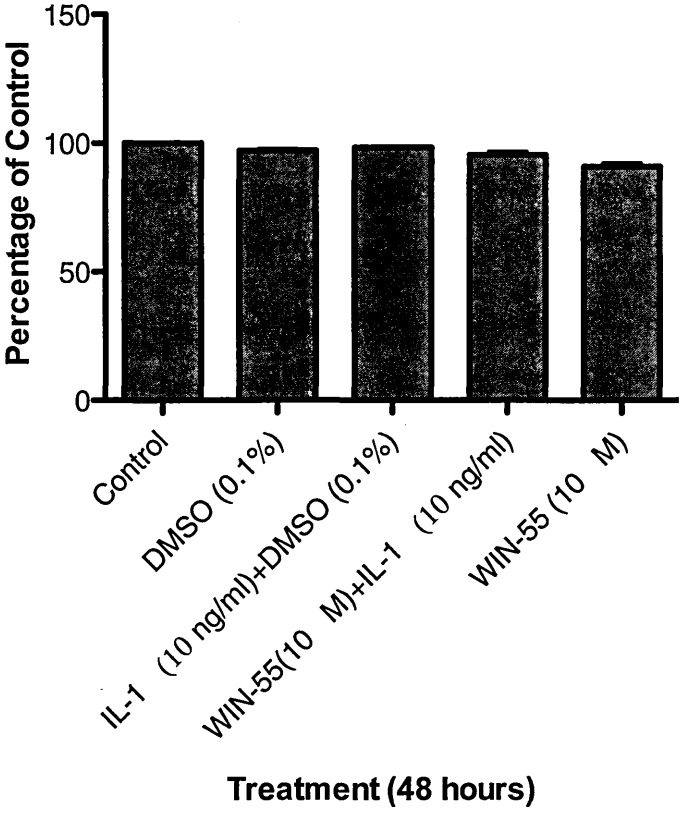

Figure 2.6 The effects of WIN-55 on chondrocyte viability. n=6 obtained from 2 patient samples. Data represents mean percentage of control under treatment conditions ± SEM.

2.6.2 The Time-Dependent Effects of WIN-55 on MMP-3, -13, TIMP-1 and -2 Gene Expression

Following WIN-55 treatment for 3 hours there was a significant increase in MMP-3 expression compared to DMSO control ($p<0.01$) and MMP-13 remained at basal levels (Figure 2.7A). Following 6 hours WIN-55 treatment MMP-3 and MMP-13 expression remained at basal levels. Significant decreases in MMP-3 and MMP-13 mRNA expression ($p<0.001$) were shown following 24 hour WIN-55 treatment compared to DMSO control (Figure 2.7A). The largest significant decrease in MMP-3 and MMP-13 mRNA expression was observed following WIN-55 treatment for 48 hours compared to DMSO vehicle control ($p<0.001$) (Figure 2.7A).

Following 3 hours WIN-55 treatment both TIMP-1 and TIMP-2 mRNA expression remained at basal levels (Figure 2.7B). After 6 hours of WIN-55 treatment there was a significant decrease in TIMP-2 gene expression compared to DMSO control ($p<0.05$) and TIMP-1 remained at basal levels (Figure 2.7B). Significant decreases in TIMP-1 and TIMP-2 mRNA expression ($p<0.01$) were shown following 24 hour WIN-55 treatment compared to DMSO control (Figure 2.7B). The largest significant decrease in TIMP-1 and TIMP-2 mRNA expression was observed following WIN-55 treatment for 48 hours compared to DMSO control ($p<0.001$) (Figure 2.7B).

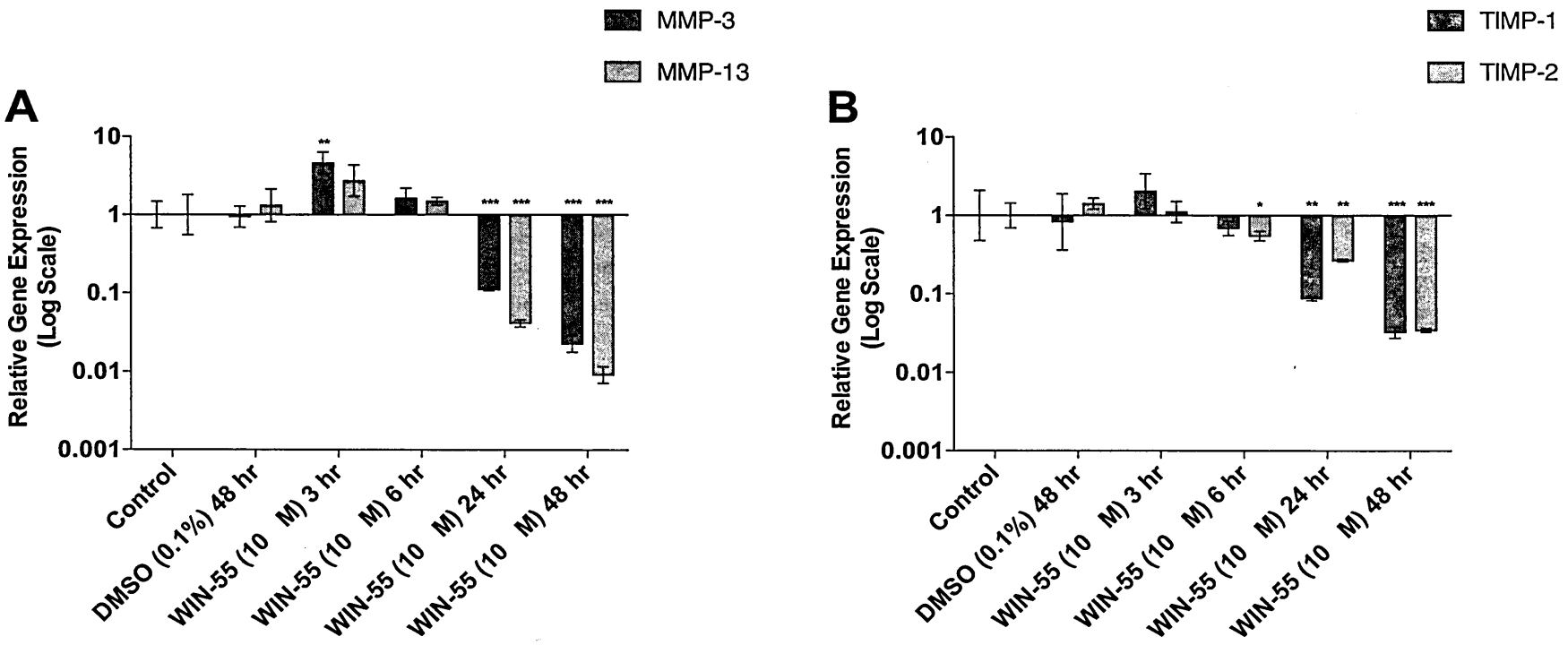

Figure 2.7 (A) The time-dependent effect of 10 μM WIN-55 on MMP-3 and -13 gene expression in OA chondrocytes cultured in monolayer. (B) The time-dependent effect of 10 μM WIN-55 on TIMP-1 and -2 gene expression in OA human chondrocytes cultured in monolayer. WIN-55 (10 μM) decreased the gene expression of MMP-3, -13, TIMP-1 and -2 in a time dependent manner, with maximal decrease obtained following 48 hours treatment. Data represents mean fold change of mRNA expression normalised to internal reference gene and untreated control ± SEM. *p<0.05, **p<0.01, ***p<0.001 compared to DMSO vehicle control. n=3 samples for each treatment group obtained from one patient sample, macroscopic grade 0 (HC20(1)).

2.6.3 The Concentration-Dependent Effects of WIN-55 on MMP-3 and -13 Gene Expression

Following IL-1β stimulation both MMP-3 and MMP-13 expression was significantly increased ($p<0.001$) (Figure 2.8). IL-1β stimulation in combination with 1 μM WIN-55 had no effect on MMP-3 and MMP-13 mRNA expression. IL-1β stimulation in combination with 2.5 μM WIN-55 had no effect on MMP-3 mRNA expression but significantly reduced MMP-13 mRNA expression below basal levels compared to DMSO alone ($p<0.05$) and IL-1β stimulation alone ($p<0.001$) (Figure 2.8). IL-1β stimulation in combination with 5 μM WIN-55 significantly reduced MMP-3 and MMP-13 mRNA expression below basal levels compared to DMSO alone and IL-1β alone ($p<0.001$) (Figure 2.8). IL-1β stimulation in combination with 7.5 μM WIN-55 significantly reduced MMP-3 mRNA expression below basal levels compared to DMSO alone ($p<0.05$) and IL-1β alone ($p<0.001$) and MMP-13 mRNA expression below basal levels compared to DMSO alone and IL-1β alone ($p<0.001$) (Figure 2.8). Following 10 μM WIN-55 treatment in combination with IL-1β stimulation there was a significant decrease in MMP-3 mRNA expression to basal levels compared to IL-1β stimulation alone ($p<0.001$) and MMP-13 mRNA expression was significantly reduced below basal levels compared to both DMSO alone and IL-1β stimulation alone ($p<0.001$) (Figure 2.8). MMP-3 mRNA expression remained at basal levels following 1 μM WIN-55 treatment and MMP-13 mRNA expression was significantly decreased to below basal levels compared to DMSO alone ($p<0.05$) (Figure 2.8). Both MMP-3 and MMP-13 mRNA levels were significantly reduced below basal levels following 2.5, 5, 7.5 and 10 μM WIN-55 treatment alone compared to DMSO alone ($p<0.001$) (Figure 2.8).

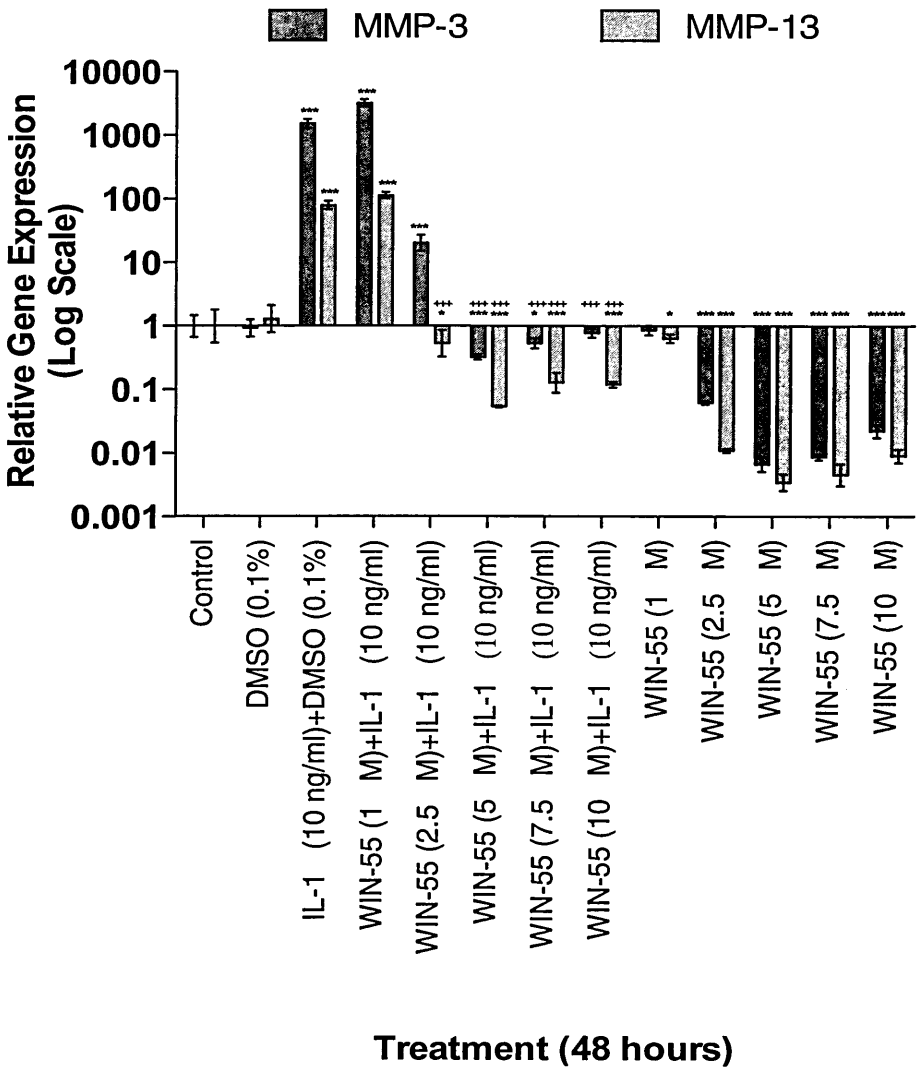

Figure 2.8 The concentration-dependent effects of WIN-55 on IL-1β induced MMP-3 and -13 gene expression in OA chondrocytes cultured in monolayer. IL-1β induced the gene expression of MMP-3 and -13. WIN-55 decreased the expression of both MMP-3 and -13 in a concentration dependent manner both alone and in combination with IL-1β. Data represents mean fold change of mRNA expression normalised to internal reference gene and untreated control ± SEM.*$p<0.05$, ***$p<0.001$ compared to DMSO control and +++$p<0.001$ compared to IL-1β treatment. n=3 samples for each treatment group obtained from one patient sample, macroscopic grade 0 (HC20(1)).

2.6.4 The Concentration-Dependent Effects of WIN-55 on TIMP-1 and -2 Gene Expression.

IL-1β stimulation had no effect on TIMP-1 and TIMP-2 mRNA expression. IL-1β stimulation in combination with 1 μM WIN-55 significantly reduced TIMP-1 mRNA expression compared to DMSO alone ($p<0.05$) and IL-1β stimulation alone ($p<0.01$) and TIMP-2 mRNA expression compared to DMSO alone ($p<0.001$) and IL-1β stimulation alone ($p<0.01$) (Figure 2.9). IL-1β stimulation in combination with 2.5, 5, 7.5 and 10 μM WIN-55 significantly reduced both TIMP-1 and TIMP-2 mRNA expression below basal levels compared to DMSO alone and IL-1β stimulation alone ($p<0.001$) (Figure 2.9). Following 1 μM WIN-55 treatment alone both TIMP-1 and TIMP-2 mRNA expression remained at basal levels (Figure 2.9). Following 2.5, 5, 7.5 and 10 μM WIN-55 treatment both TIMP-1 and TIMP-2 mRNA expression was significantly reduced below basal levels compared to DMSO alone ($p<0.001$) (Figure 2.9).

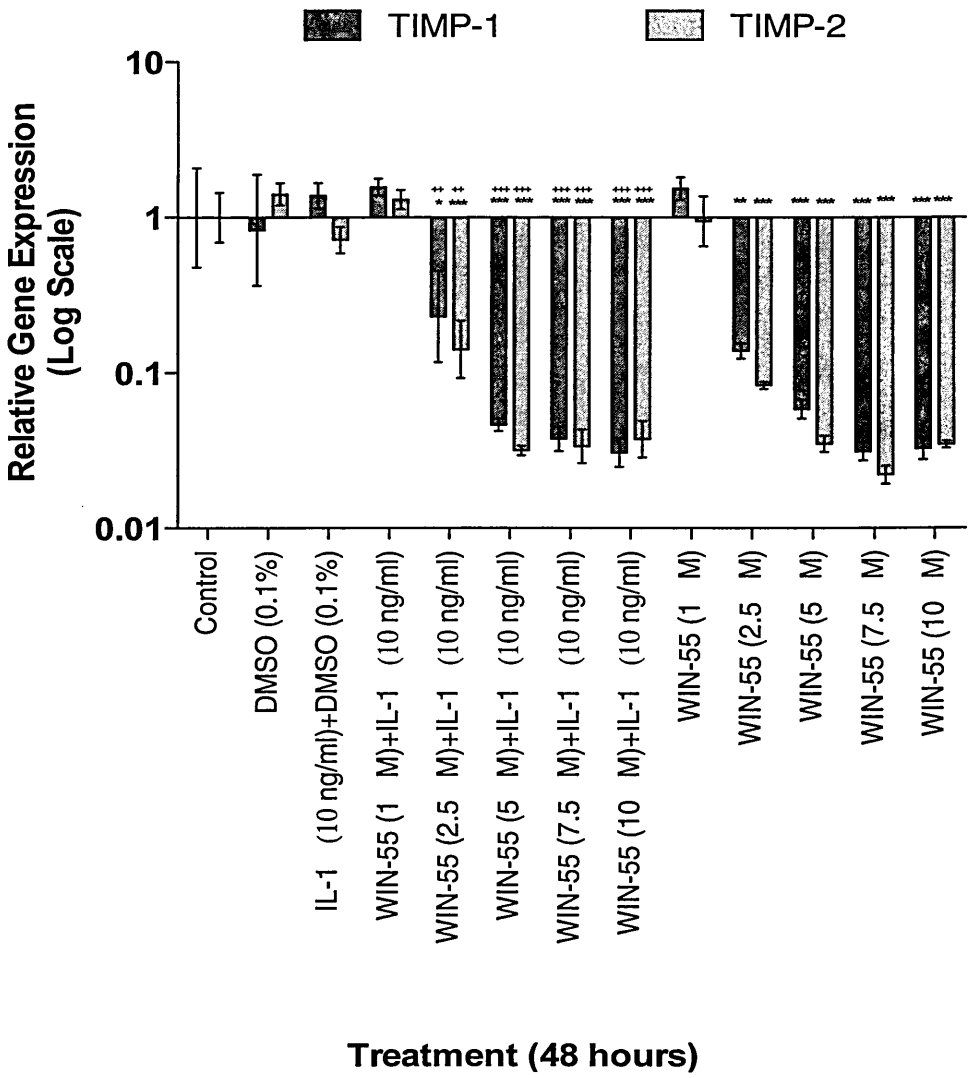

Figure 2.9 The concentration-dependent effects of WIN-55 and IL-1β on TIMP-1 and -2 gene expression in OA chondrocytes cultured in monolayer. IL-1β had no effect on TIMP-1 and -2. TIMP-1 and -2 gene expression is decreased in a WIN-55 concentration dependent manner both alone and in combination with IL-1β. Data represents mean fold change of mRNA expression normalised to internal reference gene and untreated control ± SEM. *p<0.05, **p<0.01, ***p<0.001 compared to DMSO control and ++p<0.01, +++p<0.001 compared to IL-1β treatment. n=3 samples for each treatment group obtained from one patient sample, macroscopic grade 0 (HC20(1)).

2.6.5 The Effects of WIN-55 on IL-1β Induced MMP-3 and MMP-13 mRNA Expression in Chondrocytes Cultured in Monolayer

In monolayer cultures IL-1β stimulation significantly induced MMP-3 gene expression in chondrocytes isolated from grade 0, 2 and 3 cartilage ($p<0.001$) and MMP-13 in chondrocytes isolated from grade 0 ($p<0.001$), grade 2 ($p<0.05$) and grade 3 cartilage ($p<0.001$) compared to DMSO vehicle control (Figure 2A & 2B). Treatment with WIN-55 in combination with IL-1β significantly reduced MMP-3 and -13 gene expression in chondrocytes derived from grade 0, 2 and 3 cartilage compared to IL-1β stimulation alone ($p<0.001$) (Figure 2A & 2B). WIN-55 treatment in combination with IL-1β also significantly reduced MMP-3 gene expression in chondrocytes isolated from grade 0, 2 and 3 cartilage and MMP-13 gene expression in chondrocytes isolated from grade 0 ($p<0.001$), grade 2 ($p<0.01$) and grade 3 ($p<0.001$) compared to DMSO vehicle control ($p<0.001$) (Figure 2A & 2B). WIN-55 treatment alone significantly reduced MMP-3 gene expression in grade 0, 2 and 3 cartilage derived chondrocytes below basal levels compared to DMSO vehicle control ($p<0.001$) (Figure 2A). WIN-55 treatment alone also significantly decreased MMP-13 gene expression in grade 0 and 3 cartilage derived chondrocytes compared to DMSO control ($p<0.001$) (Figure 2A). MMP-13 was only expressed in two samples in chondrocytes derived from grade 2 cartilage following WIN-55 treatment therefore statistical analysis could not be performed.

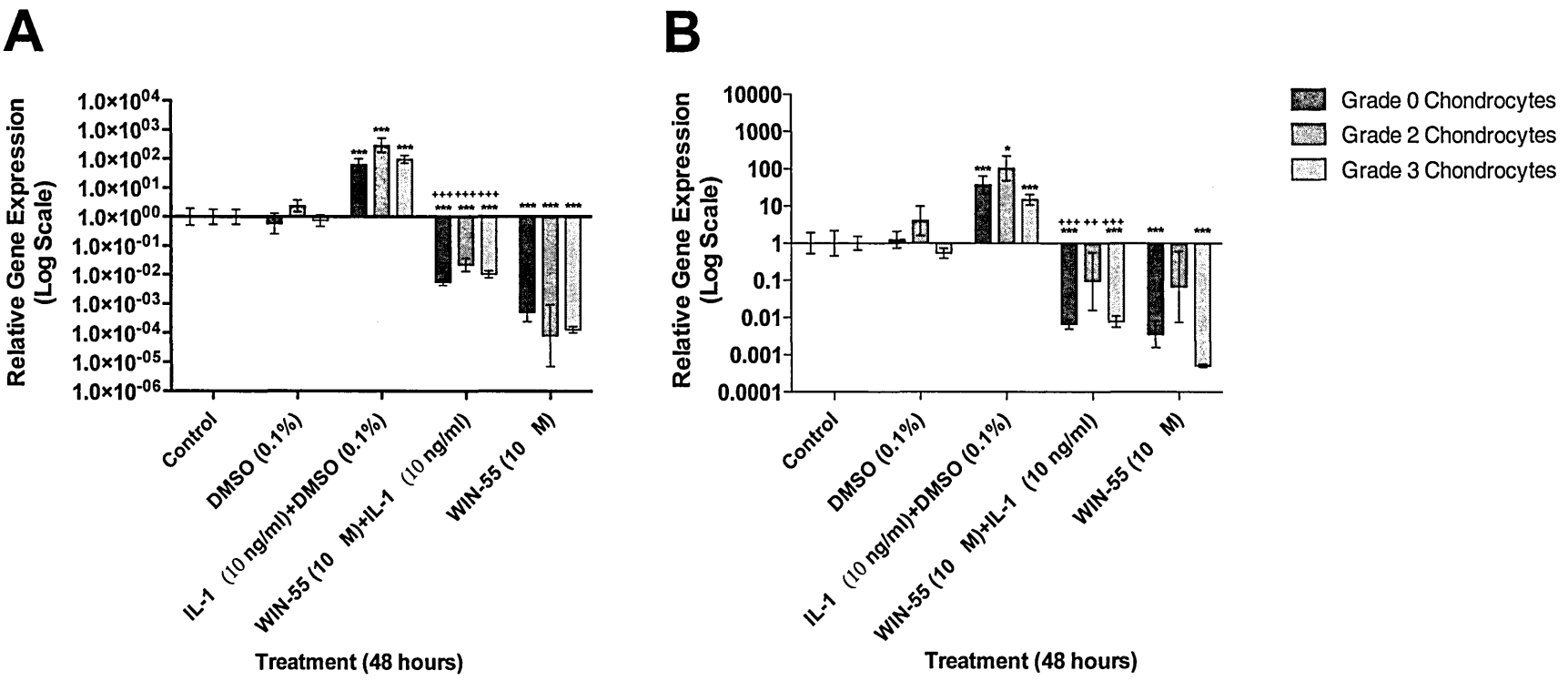

Figure 2.10 (A) The effect of WIN-55 on IL-1β induced MMP-3 gene expression in OA chondrocytes cultured in monolayer. (B) The effect of WIN-55 on IL-1β induced MMP-13 gene expression in OA chondrocytes cultured in monolayer. IL-1β induced the gene expression of MMP-3 and -13. Following WIN-55 treatment in combination with IL-1β MMP-3 and -13 gene expression is decreased. WIN-55 (10 μM) alone decreased MMP-3 and -13 gene expression. Data represents mean fold change of mRNA expression normalised to internal reference gene and untreated control ± SEM. *p<0.05, **p<0.01, ***p<0.001 compared to DMSO control and ++p<0.01, +++p<0.001 compared to IL-1β treatment. n=9 samples for each treatment group, obtained from 3 patient samples for each grade of cartilage.

2.6.6 The Effects of WIN-55 on IL-1β Induced MMP-3 and MMP-13 mRNA Expression in Human Chondrocytes Cultured in Alginate Beads

IL-1β stimulation of cells cultured in alginate beads significantly induced MMP-3 mRNA expression in chondrocytes from grade 0, 2 and 3 cartilage ($p<0.001$) and MMP-13 mRNA expression in grade 0 ($p<0.05$), 2 and 3 ($p<0.001$) cartilage derived chondrocytes compared to DMSO control (Figure 11A & 11B). Similarly WIN-55 treatment in combination with IL-1β significantly reduced MMP-3 mRNA expression in grade 0, 2 and 3 cartilage derived chondrocytes ($p<0.001$) and MMP-13 mRNA expression in grade 0 and 3 cartilage derived chondrocytes compared to IL-1β stimulation alone ($p<0.001$) (Figure 11A & 11B). MMP-13 was not expressed in chondrocytes from grade 2 cartilage treated with IL-1β in combination with WIN-55 (Figure 11B). WIN-55 treatment in combination with IL-1β significantly reduced MMP-3 ($p<0.05$) and MMP-13 ($p<0.01$) mRNA expression in chondrocytes from grade 0 chondrocytes compared to DMSO control (Figure 11A & 11B). WIN-55 treatment in combination with IL-1β reduced MMP-3 mRNA expression in chondrocytes from grade 2 cartilage and both MMP-3 and MMP-13 mRNA expression in chondrocytes from grade 3 cartilage compared to DMSO control; however this was not significant (Figure 11A & 11B). There was no significant difference from basal levels of MMP-3 mRNA expression in grade 0 and 3 cartilage derived chondrocytes when treated with WIN-55 alone (Figure 11A). MMP-3 was not expressed in grade 2 cartilage chondrocytes treated with WIN-55 alone (Figure 11A). MMP-13 was abolished in chondrocytes from grade 0, 2 and 3 cartilage treated with WIN-55 alone (Figure 11B).

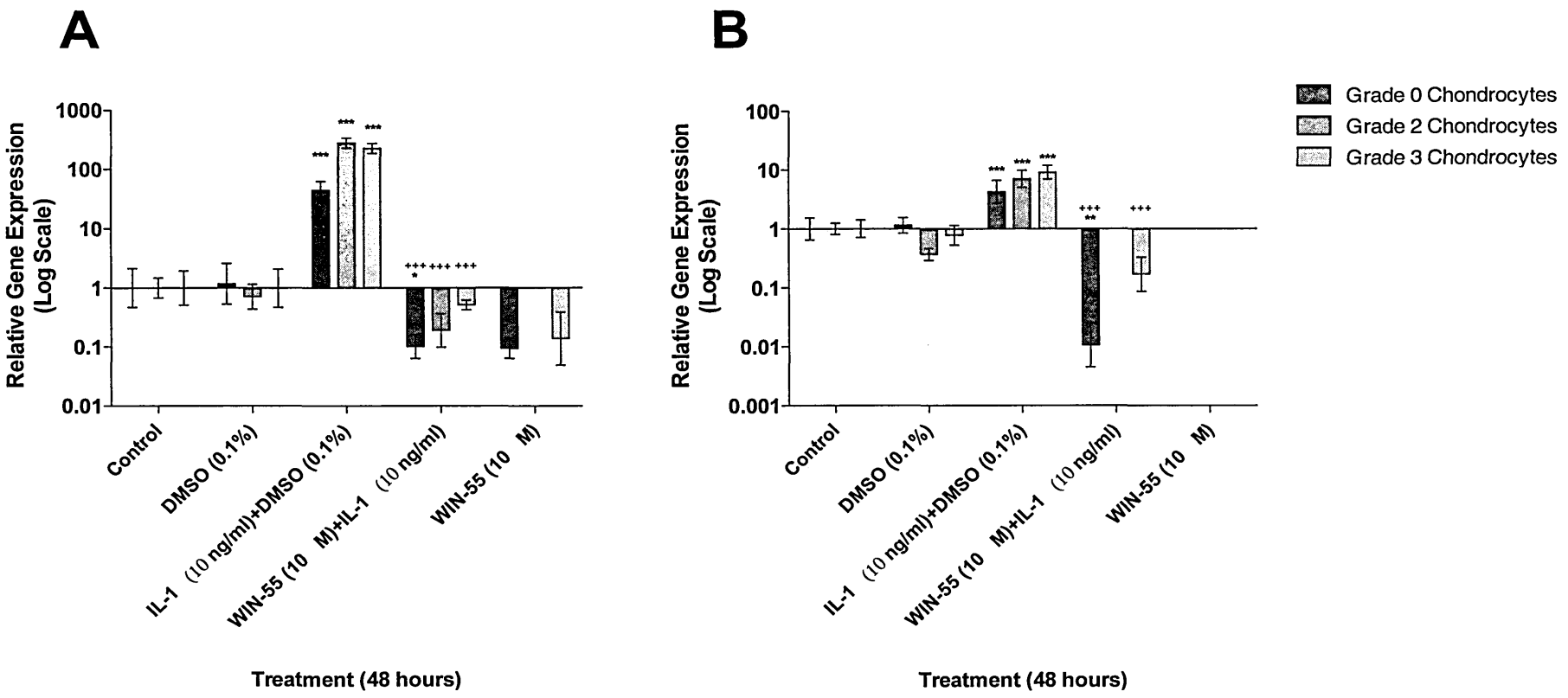

Figure 2.11 (A) The effect of WIN-55 on IL-1β induced MMP-3 gene expression in OA chondrocytes cultured in alginate beads. (B) The effect of WIN-55 on IL-1β induced MMP-13 gene expression in OA chondrocytes cultured in alginate beads. IL-1β induces the gene expression of MMP-3 and -13. Following WIN-55 treatment in combination with IL-1β MMP-3 and -13 gene expression is decreased or abolished. WIN-55 alone decreases or abolishes MMP-3 and -13 gene expression. Data represents mean fold change of mRNA expression normalised to internal reference gene and untreated control ± SEM.*p<0.05, **p<0.01, ***p<0.001 compared to DMSO control and +++p<0.001 compared to IL-1β treatment. n=9 samples for each treatment group, obtained from 3 patient samples for each grade of tissue.

2.6.7 The Effects of WIN-55 on IL-1β Induced MMP-3 and MMP-13 Gene Expression in Chondrocytes Cultured in Cell Pellets

IL-1β stimulation of cells cultured in pellets significantly induced MMP-3 mRNA expression in chondrocytes isolated from grade 0, 2 and 3 ($p<0.001$) cartilage and MMP-13 mRNA expression in chondrocytes isolated form grade 0 ($p<0.01$) and 3 ($p<0.001$) but not grade 2 cartilage compared to DMSO alone (Figure 2.12A & B). WIN-55 treatment in combination with IL-1β significantly reduced MMP-3 mRNA expression in chondrocytes isolated from grade 0, 2 and 3 ($p<0.001$) cartilage and MMP-13 mRNA expression in chondrocytes isolated from grade 0 ($p<0.01$), and 2 ($p<0.05$) cartilage compared to IL-1β stimulation alone (Figure 2.12A & B). WIN-55 treatment in combination with IL-1β significantly reduced MMP-3 mRNA expression in chondrocytes isolated from grade 0 ($p<0.001$), 2 ($p<0.05$) and 3 ($p<0.001$) cartilage below basal levels compared to DMSO control and reduced MMP-13 mRNA expression in grade 0 and grade 2 isolated chondrocytes compared to DMSO control; however this was not significant (Figure 2.12A & B). MMP-13 mRNA was not expressed in grade 3 chondrocytes treated with WIN-55 in combination with IL-1β (Figure 2.12B). WIN-55 treatment alone significantly reduced MMP-3 mRNA expression ($p<0.001$) in chondrocytes extracted from grade 2 cartilage below basal levels compared to DMSO control (Figure 2.12A). WIN-55 treatment alone reduced MMP-13 gene expression in grade 2 chondrocytes; however this was not significant (Figure 2.12B). Both MMP-3 and MMP-13 mRNA was not expressed in chondrocytes isolated form grade 0 and 3 cartilage when treated with WIN-55 alone (Figure 2.12A & B).

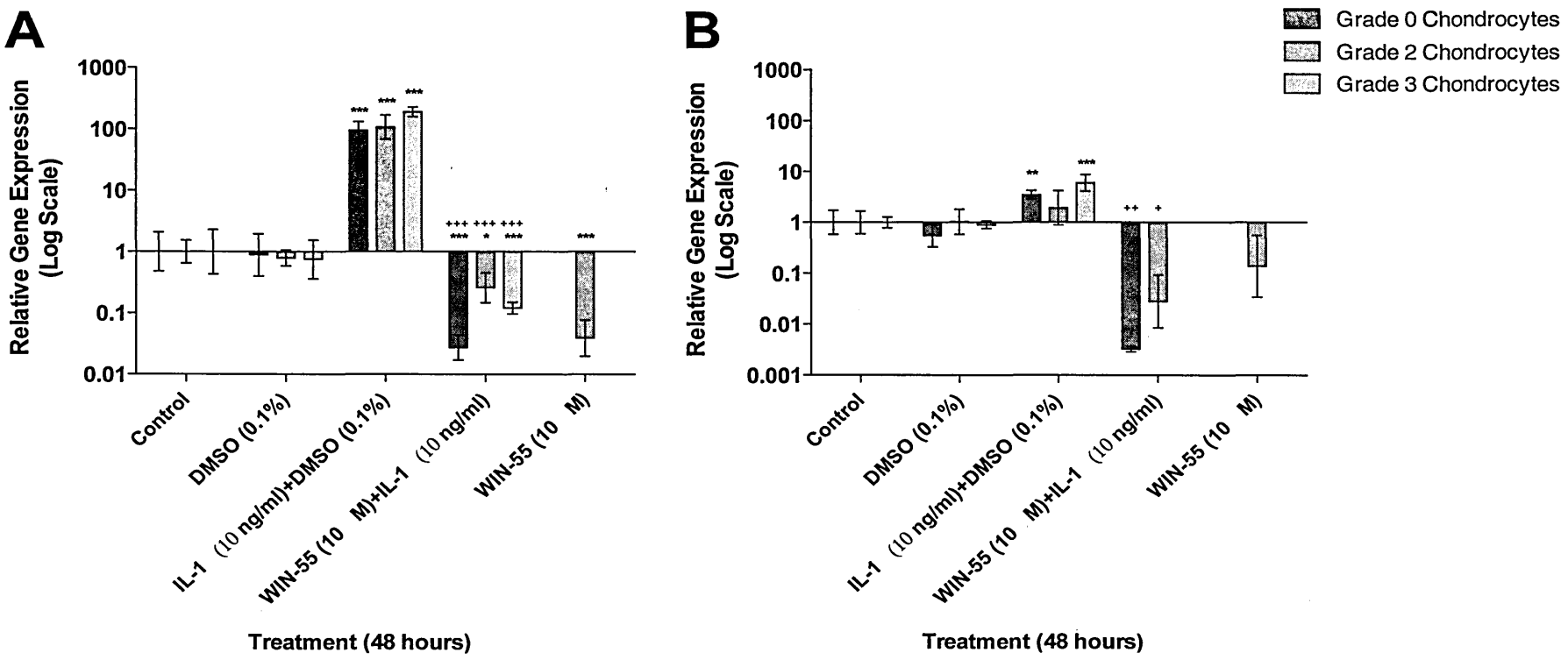

Figure 2.12 (A) The effect of WIN-55 on IL-1β induced MMP-3 gene expression in OA chondrocytes cultured in cells pellets. (B) The effect of WIN-55 on IL-1β induced MMP-13 gene expression in OA chondrocytes cultured in cell pellets. IL-1β induced the gene expression of MMP-3 in chondrocytes isolated from grade 0, 2 and 3 cartilage and MMP-13 gene expression in chondrocytes from grade 0 and 3 cartilage but not grade 2 isolated chondrocytes. WIN-55 in combination with IL-1β decreased or abolished MMP-3 and MMP-13 gene expression. WIN-55 treatment alone also decreased or abolished both MMP-3 and -13 gene expression. Data represents mean fold change of mRNA expression normalised to internal reference gene and untreated control ± SEM.*p<0.05, **p<0.01, ***p<0.001 compared to DMSO control and +p<0.05, ++p<0.01 and +++p<0.001 compared to IL-1β treatment. n=9 samples for each treatment group, obtained from 3 patient samples for each grade of tissue.

2.6.8 The Effects of WIN-55 on TIMP-1 and 2 mRNA Expression in Chondrocytes Cultured in Monolayer

In cells cultured in monolayer, IL-1β stimulation had no significant effect on TIMP-1 and TIMP-2 mRNA expression in chondrocytes derived from grade 0, 2 and 3 cartilage (Figure 2.13A & B). However WIN-55 treatment in combination with IL-1β resulted in a significant decrease in TIMP-1 gene expression compared to DMSO control and IL-1β stimulated chondrocytes isolated from grade 0 ($p<0.001$), 2 ($p<0.001$) and 3 cartilage ($p<0.01$) (Figure 2.13A). WIN-55 alone also significantly reduced TIMP-1 mRNA expression in chondrocytes derived from grade 0 and 2 cartilage ($p<0.001$) below basal levels (Figure 2.13A). TIMP-1 mRNA expression was decreased in chondrocytes derived from grade 3 cartilage following WIN-55 treatment however this was not significant (Figure 2.13A). TIMP-2 mRNA expression was significantly reduced following WIN-55 treatment in combination with IL-1β compared to DMSO control and IL-1β stimulation in chondrocytes derived from grade 0, 2 and 3 cartilage ($p<0.001$) (Figure 2.13B). WIN-55 alone significantly reduced the mRNA expression of TIMP-2 below basal levels in chondrocytes isolated from grade 0, 2 and 3 cartilage ($p<0.001$) (Figure 2.13B).

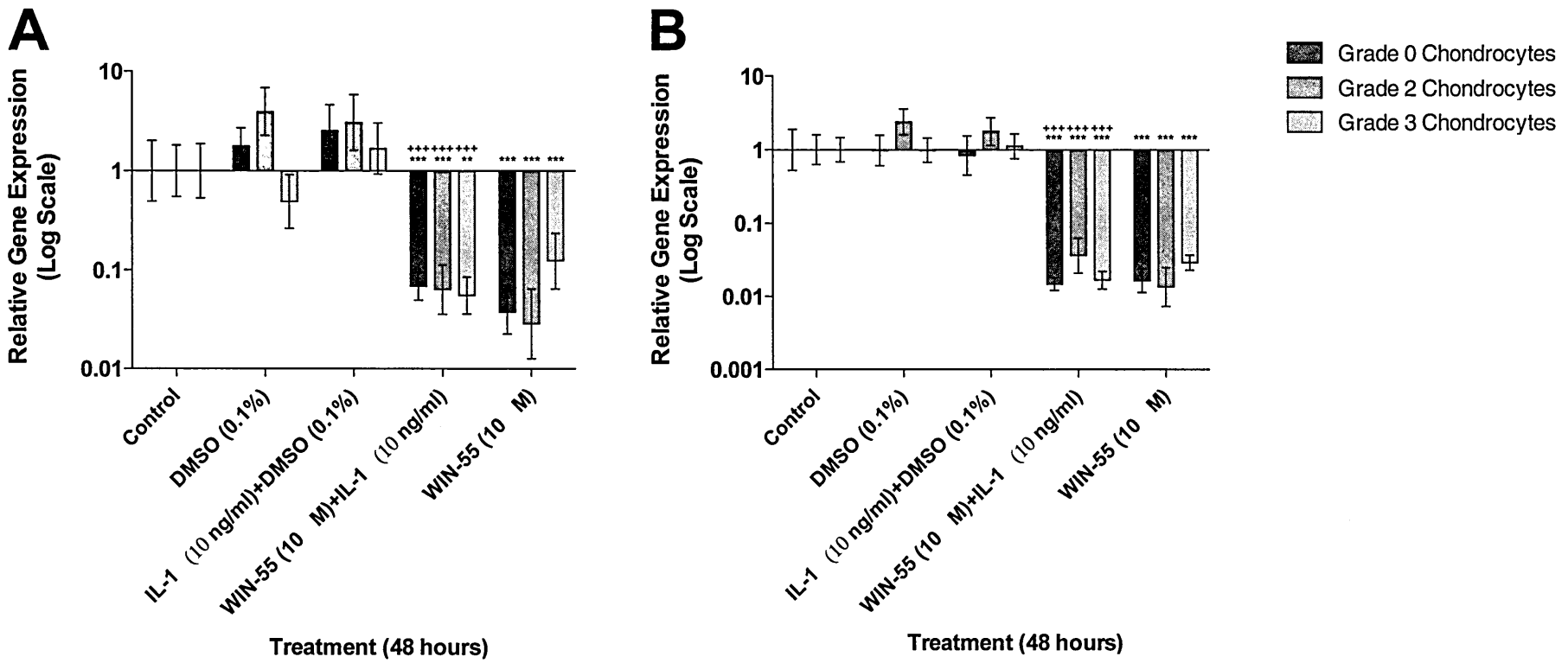

Figure 2.13 (A) The effect of WIN-55 and IL-1β on TIMP-1 gene expression in OA chondrocytes cultured in monolayer. (B) The effect of WIN-55 and IL-1β on TIMP-2 gene expression in OA chondrocytes cultured in monolayer. IL-1β had no effect on TIMP-1 and -2 gene expression. WIN-55 treatment both alone and in combination with IL-1β reduced TIMP-1 and -2 gene expression. Data represents mean fold change of mRNA expression normalised to internal reference gene and untreated control ± SEM.**p<0.01, ***p<0.001 compared to DMSO vehicle control and $^{+++}$p<0.001 compared to IL-1β treatment. N=9 samples for each treatment group, obtained from 3 patient samples for each grade of tissue.

2.6.9 The Effects of WIN-55 on TIMP-1 and -2 Gene Expression in Chondrocytes Cultured in Alginate Beads

TIMP-1 mRNA expression was significantly increased in grade 2 ($p<0.001$) and 3 cartilage derived chondrocytes ($p<0.05$) following IL-1β stimulation but not in grade 0 cartilage chondrocytes compared to DMSO control (Figure 2.14A) in cells cultured in alginate beads. In contrast TIMP-2 mRNA expression was significantly decreased following IL-1β treatment in chondrocytes extracted from grade 0 ($p<0.01$), 2 ($p<0.01$) and 3 ($p<0.001$) cartilage compared to DMSO control (Figure 2.14B). WIN-55 treatment in combination with IL-1β resulted in a significant decrease in TIMP-1 and TIMP-2 mRNA expression in chondrocytes derived from grades 0, 2 and 3 cartilage compared to IL-1β stimulation and DMSO control ($p<0.001$) (Figure 2.14A & B). WIN-55 treatment alone significantly reduced both TIMP-1 and TIMP-2 gene expression in chondrocytes derived from grade 0, 2 and 3 cartilage below basal levels compared to DMSO control ($p<0.001$) (Figure 2.14A & B).

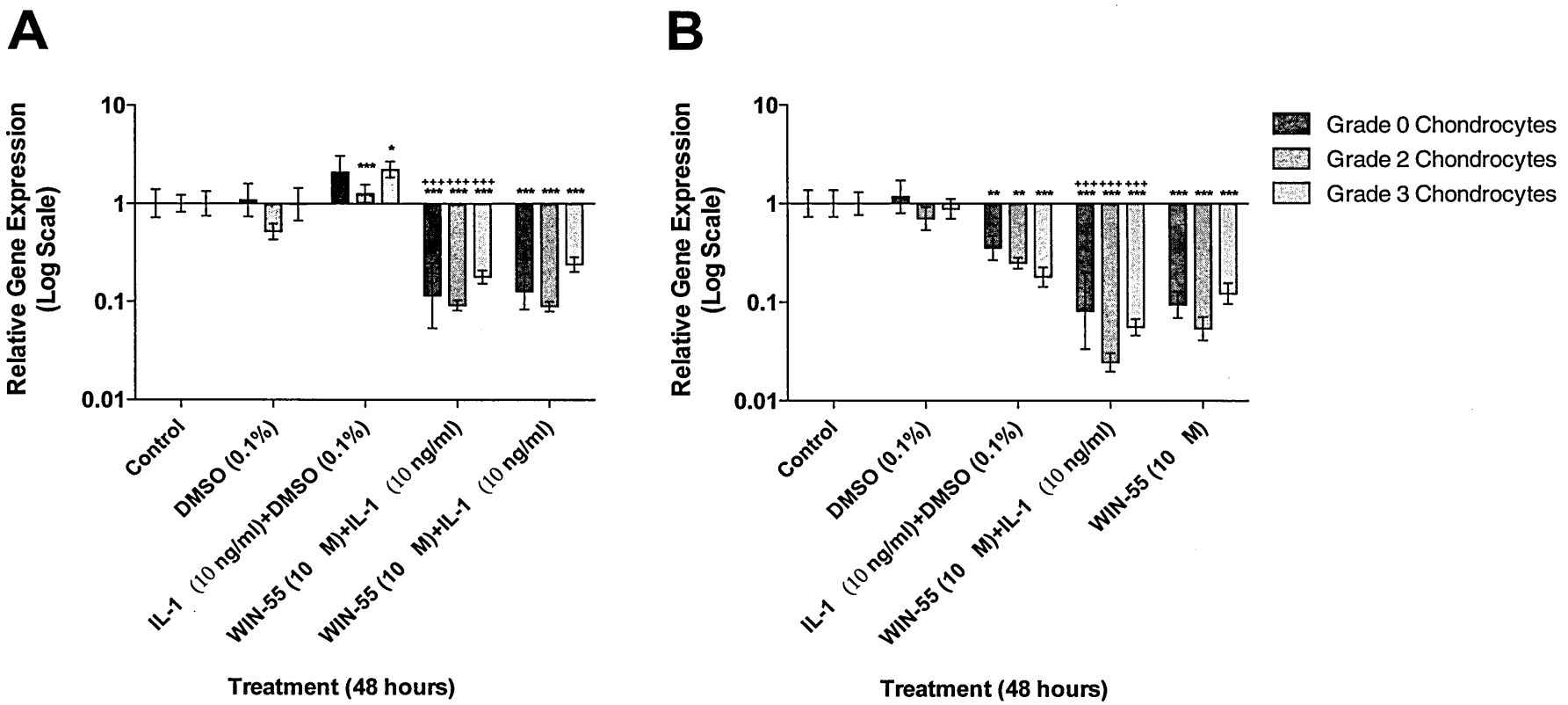

Figure 2.14 (A) The effect of WIN-55 and IL-1β on TIMP-1 gene expression in OA chondrocytes cultured in alginate beads. (B) The effect of WIN-55 and IL-1β on TIMP-2 gene expression in OA chondrocytes cultured in alginate beads. IL-1β stimulation increased TIMP-1 gene expression and decreased TIMP-2 gene expression below basal levels. WIN-55 treatment both alone and in combination with IL-1β reduced TIMP-1 and -2 gene expression. Data represents mean fold change of mRNA expression normalised to internal reference gene and untreated control ± SEM.*p<0.05, **p<0.01, ***p<0.001 compared to DMSO vehicle control and $^{+++}$p<0.001 compared to IL-1β treatment. N=9 samples for each treatment group, obtained from 3 patient samples for each grade of tissue.

2.6.10 The Effects of WIN-55 on IL-1β Induced Pro-MMP-3 Protein Release from Chondrocytes Cultured in Monolayer

Following IL-1β stimulation of cells cultured in monolayer, pro-MMP-3 release into culture media was significantly increased compared to DMSO control ($p<0.01$) (Figure 2.15A). WIN-55 treatment in combination with IL-1β significantly reduced the release of pro-MMP-3 compared to IL-1β treatment alone ($p<0.001$) and DMSO control ($p<0.01$) (Figure 2.15A). WIN-55 treatment alone significantly reduced pro-MMP-3 release from chondrocytes below basal levels compared to DMSO control ($p<0.001$) (Figure 2.15A).

2.6.11 The Effects of WIN-55 on Pro-MMP-3 and Total and Pro-MMP-13 Release From Chondrocytes Cultured in Alginate beads

Following stimulation of chondrocytes cultured in alginate beads with IL-1β there was a significant increase in MMP-3 ($p<0.001$) and MMP-13 ($p<0.05$) protein release into the media compared to DMSO control (Figure 2.15B & C). Treatment of chondrocytes with WIN-55 in combination with IL-1β significantly reduced both MMP-3 and MMP-13 protein compared to IL-1β treatment alone ($p<0.001$) (Figure 2.15B & C). WIN-55 treatment alone significantly reduced MMP-3 protein release to below basal levels compared to DMSO control ($p<0.05$) and MMP-13 protein levels remained at basal level (Figure 2.15B & C)

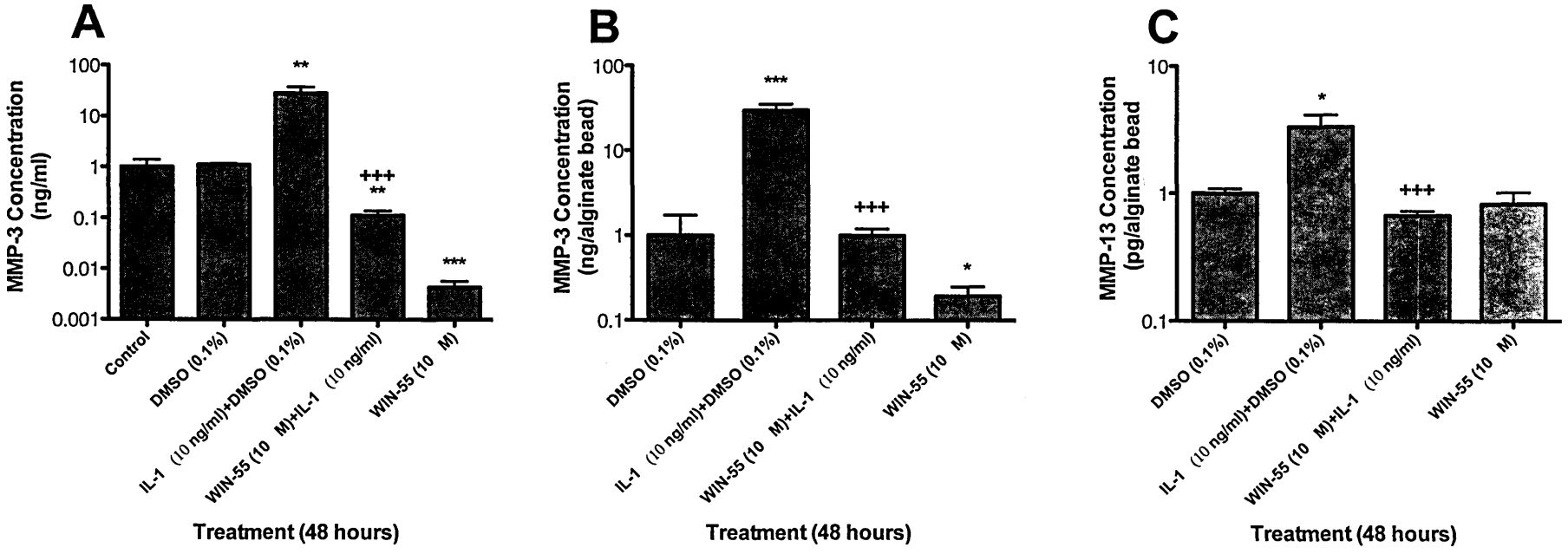

Figure 2.15 (A) The effects of WIN-55 on IL-1β induced MMP-3 protein release from monolayer, (B&C) MMP-3 and -13 protein release from alginate beads IL-1β increases the release of MMP-3 and -13 from monolayer or alginate beads. WIN-55 in combination with IL-1β decreases the release of MMP-3 and -13 from monolayer or alginate beads. WIN-55 alone decreased MMP-3 release from monolayer or alginate bead below basal level and MMP-13 release from alginate bead remained at basal levels. Data represents mean fold change of protein expression normalised to untreated control ± SEM.*$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to DMSO control and +++$p<0.001$ compared to IL-1β treatment alone. n=3 obtained from one grade 3 cartilage patient for monolayer culture and N=7 from three grade 3 cartilage patients for alginate bead culture.

2.6.12 The Effects of WIN-55 on IL-1β Induced MMP-3 Activity

The optimal incubation time was determined to be between 60 (R_1) and 300 (R_2) minutes (Figure 2.16A). MMP-3 enzyme activity remained at similar levels following stimulation with IL-1β and WIN-55 both alone and in combination with IL-1β having no significant effect on MMP-3 enzyme activity, compared to DMSO control (Figure 2.16B). Levels of MMP-3 activity were low in culture media.

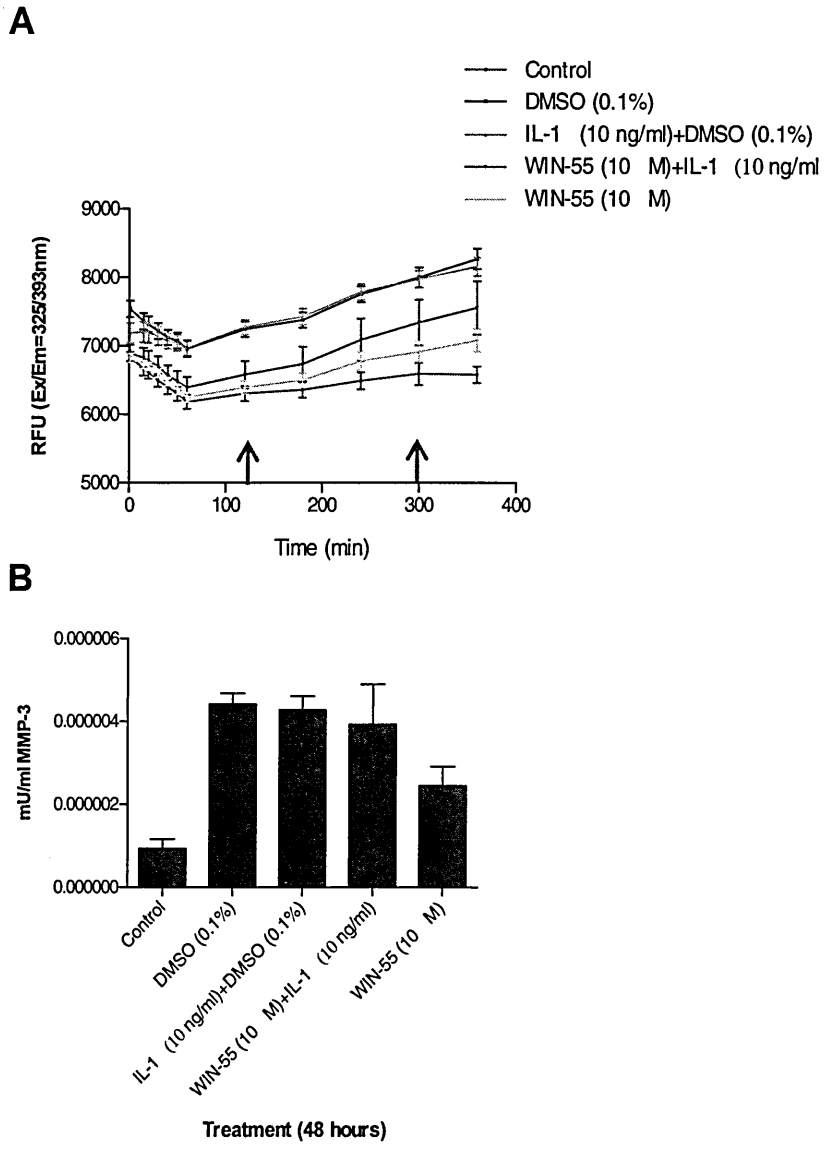

Figure 2.16 The effect of WIN-55 on MMP-3 enzyme activity in monolayer culture media. (A) The exponential phase of the reaction time for MMP-3 activity was determined to be between 120 and 300 minutes (red arrows). **(B)** MMP-3 enzyme activity in chondrocytes cultured in monolayer. Data represents mean enzyme activity ± SEM. n=3 obtained from one macroscopic grade 2 cartilage sample (HC5(4)).

2.7 Discussion

The aim of this study was to determine the effects of synthetic cannabinoid WIN-55 on the expression of MMPs and TIMPs in the presence of the catabolic cytokine IL-1β, in different grades of macroscopically grade OA cartilage.

The present study demonstrated that treatment of articular chondrocytes from human OA cartilage with synthetic cannabinoid WIN-55 reduced or abolished the mRNA or protein expression of MMP-3 and MMP-13 in the presence of IL-1β. Cartilage degradation is a pathological feature of both OA and RA (Goldring and Marcu 2009) and the catabolic cytokine IL-1β plays a key role in cartilage destruction and stimulates increased production of MMPs by chondrocytes, resulting in the breakdown of collagen and proteoglycan (Burrage *et al,* 2006). Reduction or abolition of MMP-3 and -13 expression by cannabinoids may be a mechanism by which they may protect against cartilage damage.

Both monolayer and 3D culture systems for culture of chondrocytes were used. Chondrocytes that have been isolated from articular cartilage dedifferentiate in monolayer culture changing their matrix synthesis, with a decrease in type II collagen and aggrecan synthesis and developing a fibroblast like phenotype and an increase in collagen type I (Mayne *et al,* 1976; von der Mark *et al,* 1977; Benya *et al,* 1978). Dedifferentiation can be reversed with the key phenotypic features of chondrocytes being preserved when cultured in a 3D system such as alginate beads and cell pellets (Caron *et al,* 2012). In this study, chondrocytes were treated with IL-1β to mimic inflammatory processes in an *in vitro* model of OA (Goldring 2000). IL-1β increased both MMP-3 and -13 mRNA and protein expression by cells in both monolayer and 3D culture of alginate beads and MMP-3 and -13 mRNA expression in cell pellets, however MMP-3 gene expression was more responsive to IL-1β in all the culture systems. MMP-3 is expressed at higher levels compared to MMP-13 *in vivo* (Bau *et al,* 2002). In addition the response of MMP-13 gene expression to IL-1β shown here in alginate beads and cell pellets was lower compared to monolayer. The varying expression levels of MMP-3 and MMP-13 following IL-1β stimulation are comparable with observations in other studies where IL-1β has been used to stimulate human cartilage explants obtained from OA cartilage, used to emulate *in vivo* conditions (Clockaerts *et al,* 2011). Factors that may contribute to

varying MMP expression *in vivo* include; the grade of cartilage tissue, the stage of the disease and the location of the chondrocytes within the different zones of cartilage (Freemont *et al,* 1997).

Only very low levels of MMP-3 activity were detected in culture media from cells in monolayer treated with IL-1β and WIN-55 alone or in combination. This may reflect actual low levels of enzyme present, even after IL-1β treatment or may be the result of loss of activities from long term storage or freezing and thawing of culture media samples retained from cell culture treatments. In addition, no activation of pro-MMP-3 was carried out by treatment of samples with activating agents such as aminophenylmercuric acetate (APMA). This requires further investigation.

During OA there is thought to be an imbalance between MMP and TIMP expression, which in part contributes to cartilage breakdown(Dean *et al,* 1989). Gene expression profiling of cells directly extracted from human OA cartilage have shown that TIMP-1 is decreased with no significant changes in TIMP-2 gene expression, in contrast TIMP-2 is up-regulated with no significant changes in TIMP-1 gene expression in cells directly extracted from OA synovium (Davidson *et al,* 2006). TIMPs are regulated at the transcription level by cytokines including IL-1β and in human RA fibroblast like synovial cells IL-1β induces TIMP-1 mRNA expression (Vincenti 2001; Page *et al,* 2010). In contrast, here it was shown that IL-1β stimulation has no effect on TIMP-1 and TIMP-2 gene expression in human OA chondrocytes cultured in monolayer. Interestingly when chondrocytes were re-differentiated in alginate beads there was a significant increase in TIMP-1 gene expression in chondrocytes isolated from grade 2 and 3 cartilage and a significant decrease in TIMP-2 gene expression below basal levels in chondrocytes extracted from grade 0, 2 and 3 cartilage in response to IL-1β stimulation, suggesting that re-differentiation of chondrocytes back to their native phenotype is an important factor when determining the response of chondrocytes to different stimuli. In addition it was shown that WIN-55 both alone and in combination with IL-1β significantly reduces the gene expression of TIMP-1 and TIMP-2 to below basal levels.

Synthetic cannabinoids WIN-55 and HU-210 reduce IL-1α induced proteoglycan and collagen degradation in bovine nasal cartilage tissue suggesting a chondroprotective effect of these compounds (Mbvundula *et al,* 2006). Here a possible mechanism by which WIN-55 may prevent IL-1β induced ECM breakdown in OA cartilage tissue was demonstrated via inhibition of MMPs at both the mRNA and protein level. In addition chondrocytes from different grades of OA cartilage were shown to modulate MMP-3 and MMP-13 expression in response to WIN-55 with and without IL-1β stimulation. These findings together with others, suggest that cannabinoids may be of importance in the treatment of arthritis (Malfait *et al,* 2000; Sumariwalla *et al,* 2004; Johnson *et al,* 2007; Selvi *et al,* 2008; Zurier *et al,* 1998). Previous *in vitro* studies demonstrated that WIN-55 and CB1 receptor agonist CP55,940 inhibited IL-1β induced secretion of IL-6 and IL-8 in RA fibroblast like synovial cells, suggesting an anti-inflammatory activity of cannabinoids (Selvi *et al,* 2008) and non-psychoactive cannabinoid AJA reduced MMP-1, MMP-3 and MMP-9 release from fibroblast like synovial cells stimulated with IL-1α and TNFα (Johnson *et al,* 2007). *In vivo*, AJA has also been shown to reduce the severity of adjuvant-induced arthritis (Zurier *et al,* 1998) and other non-psychoactive cannabinoids, CBD and HU-320 reduced inflammation and joint damage in murine collagen-induced arthritis (Malfait *et al,* 2000; Sumariwalla *et al,* 2004).

The effects of WIN-55 on articular chondrocytes did not appear to be influenced by the grade of the cartilage they were isolated from when cultured in monolayer. Chondrocytes cultured in monolayer express MMP-3 and MMP-13 at very low levels following WIN-55 treatment, but greater inhibitory effects were observed in 3D cultures. Interestingly a biphasic expression pattern of MMP-3 and MMP-13 in response to WIN-55 was observed in 3D culture of both cell pellet and alginate beads. MMP-3 and MMP-13 genes were expressed at low levels in grade 2 chondrocytes and were not expressed in grade 0 and grade 3 chondrocytes cultured in cell pellets following WIN-55 stimulation. In contrast MMP-3 was expressed in grade 0 and grade 3 chondrocytes and not in grade 2 tissue and MMP-13 was not expressed in any of the grades of chondrocytes cultured in alginate beads following WIN-55 stimulation. These varying responses to WIN-55 treatment in alginate bead culture may indicate that the

expression of MMPs may be differentially regulated depending on the grade and extent of cartilage degradation and the culture method utilised. Studies have shown that cartilage tissue derived from different OA grades or normal aged cartilage may influence the response of the chondrocytes to different treatments (Dozin *et al,* 2002; Hickery *et al,* 2003; Fan *et al,* 2005). Moreover biphasic effects have also been seen with other cannabinoids namely AJA (Burstein 2005).

The data presented shows that WIN-55 inhibits expression of both destructive MMPs and protective TIMPs involved in the pathogenesis of OA, indicating that inhibition may occur via a signalling pathway which regulates both at the transcription level. Human MMPs and TIMPs share a common AP-1 site in their promoters that regulates their transcription (Vincenti and Brinckerhoff 2002; Borden and Heller 1997). WIN-55 may have a differential effect on AP-1 activation via PPARs. WIN-55 has been shown to activate AP-1 via PPARα. In addition AP-1 may be involved in the activation of interferon β (IFNβ) (Downer *et al,* 2012). Production of IFNβ may result in reduced levels of MMPs and TIMPs as IFNβ reduced MMP-1, -3 and TIMP-1 in fibroblast-like synovial cells both with and without IL-1β stimulation and synovial tissue from patients with RA, treated with IFNβ, showed reduced levels of MMP-1 and TIMP-1 (Smeets *et al,* 2000). Furthermore IFNβ has been shown to have anti-inflammatory properties in the treatment of arthritis (Tak *et al,* 1999; van Holten *et al,* 2002; van Holten *et al,* 2004). Conversely PPARγ agonists have been shown to reduce IL-1β induced MMP-1 expression in human synovial fibroblasts via inhibiting DNA binding of AP-1 (Fahmi *et al,* 2002). WIN-55 also binds to PPARγ so could also act in this way (O'Sullivan 2007).

2.7.1 Summary

In OA chondrocytes, the synthetic cannabinoid WIN-55 inhibits the expression of matrix degrading enzymes MMP-3 and -13 both at the mRNA and protein level and their inhibitors TIMP-1 and -2 at the mRNA level in the presence or absence of inflammatory cytokine IL-1β, in a concentration and time dependent manner. This suggests a possible mechanism by which cannabinoids may act to prevent ECM breakdown in arthritis. Since TIMP-1 and -2 are also decreased

by WIN-55 in human OA chondrocytes it is unclear whether there is a change in MMP and TIMP balance following cannabinoid treatment. However the inhibitory effect of WIN-55 on MMP-3 and -13 expression would indicate a possible role of cannabinoids in supressing IL-1β induced ECM degradation by MMPs.

3 Effects of WIN-55 on other Catabolic Events Induced by IL-1β

3.1 Introduction

3.1.1 Chemokines in OA

Chemokines are known to be involved in cartilage degradation and their expression leads to the induction of MMPs by chondrocytes (Borzi *et al,* 2000;Borzi *et al,* 2004). In human and bovine chondrocytes MMP-13 secretion is up-regulated by IL-8 (Merz *et al,* 2003), suggesting IL-8 plays a role in cartilage breakdown via the upregulation of matrix degrading enzymes, thus directly contributing to cartilage breakdown. Furthermore, IL-8 induced the expression of hypertrophic markers including collagen type X in addition to cartilage calcification (Merz *et al,* 2003). The highly selective IL-8 receptor (CXCR1) is expressed at a higher level in OA cartilage compared to normal cartilage, suggesting IL-8 has direct effects on chondrocytes (Borzi *et al,* 2000). IL-1β has direct effects on chemokine production; in human articular chondrocytes IL-1β induces the production of chemokine IL-8 that is thought to contribute to cartilage breakdown and inflammation via the recruitment and degranulation of neutrophils (Lotz *et al,* 1992; Elford and Cooper 1991). Collectively, these finding suggest that IL-8 is involved in the inflammatory process of OA in addition to altered differentiation of articular chondrocytes. IL-8 is also produced by other joint cells involved in the pathogenesis of OA including, synovial cells (Kaneko *et al,* 2000). Inhibition of IL-8 production may therefore be an important target in the treatment of OA. Previous studies have shown that cannabinoids display anti-inflammatory properties and WIN-55 reduced IL-1β induced secretion of IL-8 from human OA and RA synovial like fibroblasts (Selvi *et al,* 2008) suggesting that WIN-55 displays anti-inflammatory effects in arthritic joints via the reduction of IL-8.

3.1.2 Pain Related Peptides in OA

Nerve growth factors including NGF, brain-derived neurotrophic factor (BDNF) and neurotrophin-3, -4 and -5 (NT-3, -4 and-5) and neuropeptides substance P and calcitonin gene related peptide (CGRP) have been associated with pain in OA (Walsh *et al,* 2010;Keeble and Brain 2004). The subchondral junction is the site of innervation and NGF expression has been associated with angiogenesis in human OA (Walsh *et al,* 2010). Substance P is thought to play a dual role in arthritis contributing to both nociception and inflammation (Keeble and Brain

2004; Seidel *et al,* 2013). Moreover, substance P has been associated with the progression and pathogenesis of arthritis as *in vivo* studies showed infusion of substance into the knee joint increasing the severity of arthritis (Levine *et al,* 1984). In addition endogenous levels of substance P are increased in the synovial fluid obtained from patients with OA and RA (Im *et al,* 2008).

Human chondrocytes have been shown to express NGF and substance P and in OA chondrocytes expression of NGF and substance P and their receptors high affinity receptor p140 tyrosine kinase a Trka and NK-1 respectively, are increased compared to normal chondrocytes (Iannone *et al,* 2002; Im *et al,* 2008; Iannone and Lapadula 1998; Millward-Sadler *et al,* 2003). Targeting NGF for analgesic effects in OA has recently been reviewed (Seidel *et al,* 2013).

During OA an increase in IL-1β is associated with increases in NGF and in human synovial fibroblasts IL-1β induced an increase of NGF levels (Manni *et al,* 2003; Manni and Aloe 1998). IL-1β up regulates BDNF, NT-3 and neuropilin 2 mRNA expression and NGF production in annulus pulposus cells of the intervertebral disc (Gruber *et al,* 2012). In addition IL-1β also induces the expression of substance P in human chondrocytes (Im *et al,* 2008). Collectively, these finding suggest that IL-1β plays a role in pain signalling in degeneration of cartilaginous tissue.

There is evidence to suggest that cannabinoids may have potential to be chondroprotective via inhibiting MMP-3 and -13 (Chapter 2, Dunn *et al,* 2013) however cannabinoids may also have a dual role in the treatment of OA via the inhibition of pain signals. In support of this, cannabinoids have been shown to have analgesic properties in animal models of arthritis and cannabis based medicine Sativex has analgesic effects in patients with RA (Blake *et al,* 2006; Schuelert and McDougall 2011; Smith *et al,* 1998; Cox and Welch 2004; Cox *et al,* 2007; Schuelert and McDougall 2008). In addition, WIN-55 has been shown to have analgesic activities and reduce nociception in animal models of inflammatory pain (Ebrahimzadeh and Haghparast 2011; Burgos *et al,* 2010). Moreover cannabinoids have been shown to inhibit substance P release from primary afferent terminals (Zhang *et al,* 2010). Together these finding suggest that cannabinoids may be of value in the treatment of OA pain via the inhibition

of production of pain related peptides and growth factors associated with the pathogenesis of OA.

3.2 Aims and Objectives

Aim: To investigate the effects of WIN-55 on the expression of chemokine IL-8, growth factor NGF and neuropeptide substance P in human OA chondrocytes in the presence of IL-1β.

Objectives:

- To determine the effects of WIN-55 on IL-1β induced production of chemokine IL-8 mRNA
- To determine the effects of WIN-55 on pain related neuropeptide substance P and nerve growth factor NGF mRNA in the presence IL-1β.

3.3 Experimental Design

The effects of WIN-55 on chemokine IL-8, growth factor NGF and neuropeptide substance P in OA chondrocytes were investigated. Cartilage tissue was graded macroscopically 0-4 using the Outerbridge classification (Cameron *et al,* 2003). Chondrocytes were isolated from grade 2 or 3 cartilage tissue as representative of low degenerate and intermediate degenerate cartilage tissue. Cartilage from grade 4, severe degenerate tissue, was not used in the study, as the cell yield obtained was not sufficient. Chondrocytes were cultured in monolayer and cells were stimulated with IL-1β to induce catabolic responses. Chondrocytes were also treated with WIN-55 with and without IL-1β and the gene expression of IL-8, NGF and substance P were investigated using real-time PCR.

3.4 Methodology

3.4.1 Human OA Cartilage Samples

Primary Human chondrocytes were obtained from the articular cartilage removed from patients with symptomatic OA at the time of total knee replacement as described in section 2.4.1.

3.4.2 Macroscopic Grading of Cartilage Tissue

Cartilage tissue was macroscopically graded 0-4 using the Outerbridge classification at time of surgery prior to isolation of chondrocytes (Cameron *et al,* 2003) as described in section 2.4.2.

3.4.3 Isolation of Human Chondrocytes

Human chondrocytes were isolated from cartilage as described in section 2.4.3.

3.4.4 OA Patient Samples

Chondrocytes cultures were derived from OA patient samples of macroscopic grades 2 or 3; HC5(1), HC11(3), HC15(4), HC16(4), and HC23(4) (Table 3.1). Full patient sample information can be found in Appendix 1.

Analysis Performed	Monolayer Culture	
	Grade 2	Grade 3
IL-8 mRNA Expression	HC5(1), HC23(4)	HC11(3), HC15(4), HC16(4)
Nerve growth factor mRNA Expression	HC5(1), HC23(4)	HC11(3), HC15(4), HC16(4)
Substance P mRNA Expression	HC5(1), HC23(4)	HC11(3), HC15(4), HC16(4)

Table 3.1 The patient samples used for each analysis performed on chondrocytes obtained from different macroscopic grades of OA cartilage. Full details of samples used in these investigations can be found in Appendix 1. The patient samples used for investigation of gene expression isolated from grade 2 and 3 cartilage were combined for real-time PCR analysis.

3.4.5 WIN-55 and IL-1β Treatment of OA Chondrocytes Cultured in Monolayer for Real-time PCR Analysis.

Cells were cultured in monolayer until 80% confluent before passaging as described in section 2.4.5. Chondrocytes were seeded in 6 well culture plates at a cell density of $5x10^5$ cells per well as described in section 2.4.6. Cell treatments were performed as outlined in section 2.4.7. Treatments were performed in triplicate on chondrocytes isolated from macroscopic grade 2 or grade 3 cartilage (Table 3.1).

3.4.6 RNA Extraction from Cells Cultured in Monolayer

Isolation of RNA was performed as described in section 2.4.13.

3.4.7 Reverse Transcription and Real-time PCR

RNA was reversed transcribed to cDNA as described in section 2.4.16. Taqman PCR was performed on cDNA as described in section 2.4.18. using pre-designed Taqman Gene Expression Assays (Table 3.2, Life Technologies).

3.4.8 Real-time PCR Analysis

The data obtained from chondrocytes isolated from grade 2 and 3 cartilage were combined prior to analysis (Table 3.1). Real-time PCR data was analysed using the $2^{-\Delta\Delta CT}$ (Livak and Schmittgen 2001) (section 2.4.20).

3.4.9 Statistical Analysis

Statistical analysis was performed as outlined in section 2.5.

Taqman Gene Expression Assay	Assay ID
IL-8	Hs00174103_m1
Nerve Growth Factor	Hs01113193_m1
Substance P (Tak1)	Hs00243225_m1

Table 3.2 Taqman gene expression IDs

3.5 Results

3.5.1 The Effects of WIN-55 on IL-1β Induced IL-8, NGF and Substance P mRNA Expression

3.5.1.1 IL-8

Following IL-1β stimulation for 48 hours there was a significant increase in IL-8 mRNA expression compared to DMSO control ($p<0.001$) (Figure 3.1) WIN-55 treatment in combination with IL-1β for 48 hours significantly reduced IL-8 mRNA expression compared to IL-1β treatment alone ($p<0.001$), however expression remained above basal levels compared to DMSO control ($p<0.001$) (Figure 3.1). IL-8 mRNA expression alone remained at basal levels following WIN-55 treatment for 48 hours (Figure 3.1).

3.5.1.2 NGF

Following IL-1β stimulation for 48 hours there was a significant increase in NGF mRNA expression compared to DMSO control ($p<0.001$) (Figure 3.2). WIN-55 treatment in combination with IL-1β for 48 hours significantly reduced NGF mRNA expression compared to IL-1β treatment alone ($p<0.001$), however expression remained above basal levels compared to DMSO control ($p<0.05$) (Figure 3.2). WIN-55 treatment alone for 48 hours induced a significant increase in NGF mRNA expression compared to DMSO alone ($p<0.01$) (Figure 3.2).

3.5.1.3 Substance P

Following IL-1β stimulation for 48 hours there was a significant increase in substance P mRNA expression compared to DMSO control ($p<0.001$) (Figure 3.3). WIN-55 treatment in combination with IL-1β for 48 hours significantly induced substance P mRNA expression compared to DMSO control and IL-1β treatment alone ($p<0.001$) (Figure 3.3). WIN-55 treatment alone for 48 hours induced a significant increase in substance P mRNA expression compared to DMSO alone ($p<0.001$) (Figure 3.3).

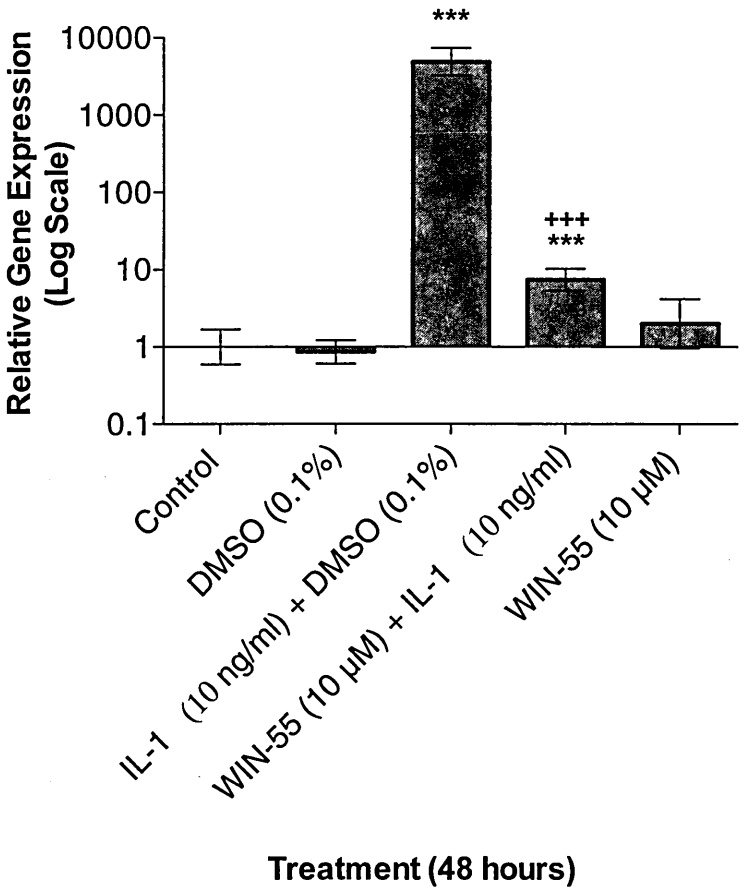

Figure 3.1 The effects of WIN-55 on IL-1β induced IL-8 gene expression. IL-1β stimulation for 48 hours significantly induced the mRNA expression of IL-8. WIN-55 treatment in combination with IL-1β for 48 hours significantly reduced IL-8 mRNA expression compared to IL-1β stimulation alone, however IL-8 gene expression remained significantly above basal levels compared to DMSO alone. Following WIN-55 treatment for 48 hours IL-8 mRNA expression remained at basal levels. Data represents mean fold change of mRNA expression normalised to internal reference gene and untreated control ± SEM. ***$p<0.001$ compared to DMSO control, +++$p<0.001$ compared to IL-1β stimulation. n=15 obtained from 5 patient samples.

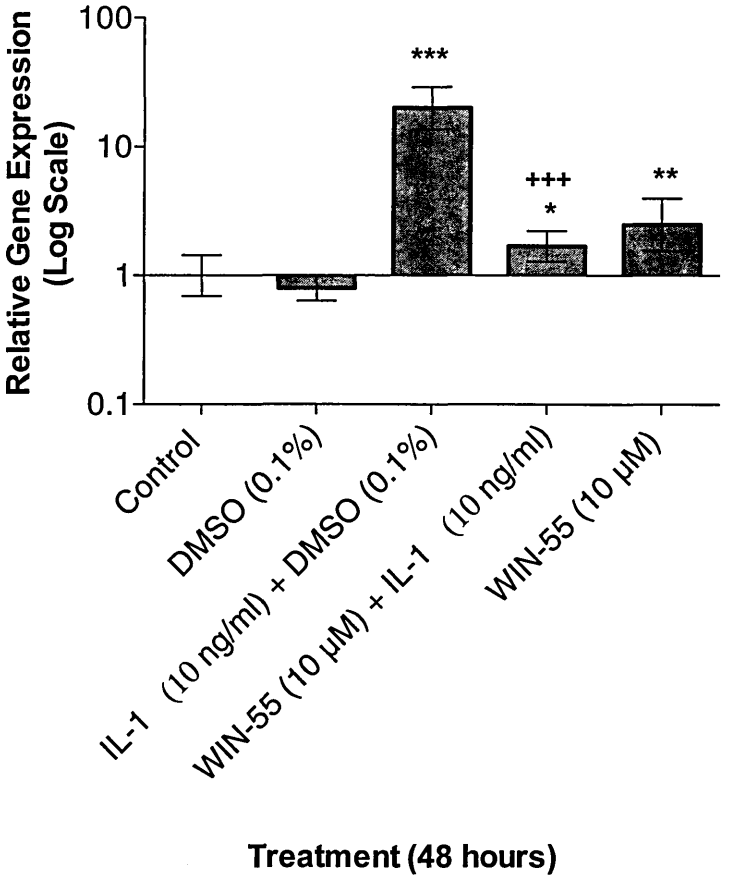

Figure 3.2 The effects of WIN-55 on IL-1β induced NGF mRNA expression. IL-1β stimulation for 48 hours induced the mRNA expression of NGF. WIN-55 treatment in combination with IL-1β for 48 hours significantly reduced NGF mRNA expression compared to IL-1β stimulation alone, however NGF mRNA expression remained significantly above basal levels compared to DMSO alone. Following WIN-55 treatment for 48 hours NGF mRNA expression was significantly increased above basal levels. Data represents mean fold change of mRNA expression normalised to internal reference gene and untreated control ± SEM. *$p<0.05$, **$p<0.01$ ***$p<0.001$ compared to DMSO control, +++$p<0.001$ compared to IL-1β stimulation. n=15 obtained from 5 patient samples.

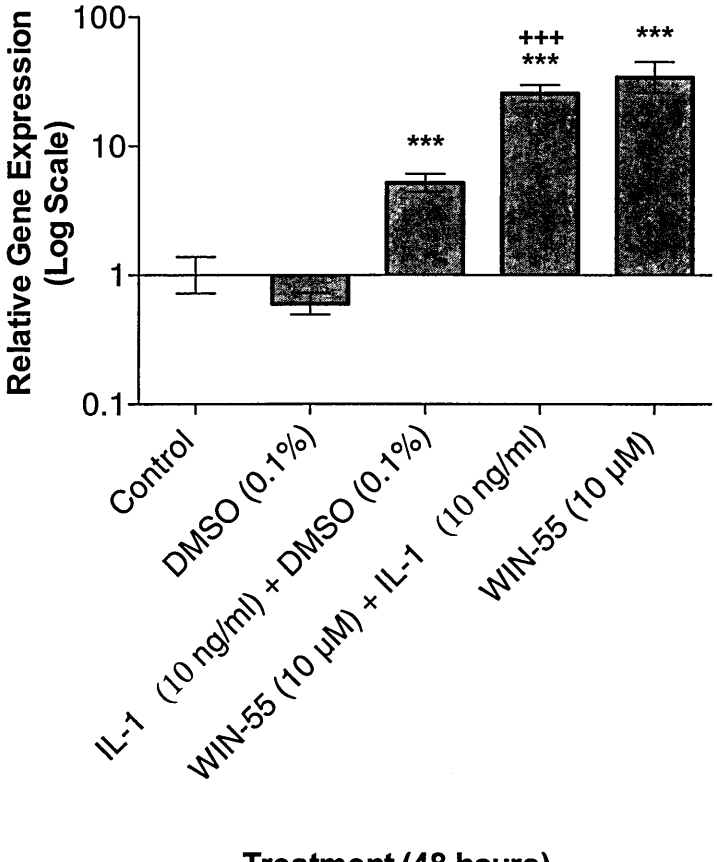

Figure 3.3 The effects of WIN-55 on IL-1β induced Substance P mRNA expression. IL-1β stimulation for 48 hours significantly induced the mRNA expression of substance P. WIN-55 treatment in combination with IL-1β for 48 hours significantly induced substance P mRNA expression compared to IL-1β stimulation alone and DMSO alone. Following WIN-55 treatment for 48 hours substance P mRNA expression was significantly increased above basal levels. Data represents mean fold change of mRNA expression normalised to internal reference gene and untreated control ± SEM. ***$p<0.001$ compared to DMSO control, +++$p<0.001$ compared to IL-1β stimulation. n=15 obtained from 5 patient samples.

3.6 Discussion

This study aimed to investigate the effects of WIN-55 on IL-1β induced IL-8 expression in human OA chondrocytes in order to determine the anti-inflammatory potential of cannabinoids during the pathogenesis of OA. In addition the effects of WIN-55 on NGF and substance P, which are thought to be involved in pain signalling pathways during the progression of OA were investigated.

3.6.1 Chemokine IL-8

IL-1β is known to induce the secretion of IL-8 from human chondrocytes subsequently inducing inflammation by neutrophil infiltration and cartilage breakdown (Lotz *et al,* 1992). Other studies support the findings presented here, that WIN-55 can inhibit IL-1β induced chemokine production. In human fibroblast-like synoviocytes obtained from patient with OA and RA, WIN-55 reduced IL-1β stimulated secretion of IL-8 (Selvi *et al,* 2008). In agreement with this study, WIN-55 treatment alone did not reduce IL-8 secretion below basal levels in both OA and RA patient samples (Selvi *et al,* 2008). In earlier studies WIN-55 inhibited TNFα induced IL-8 release via inhibiting the degradation of IκBα complex and ultimately the activation of NFκB signalling (Mormina *et al,* 2006). WIN-55 has also been shown to inhibit IL-1β induced IL-8 mRNA expression via blocking IL-1β activation of its promoter (Curran *et al,* 2005).

Studies have shown that IL-8 has binding sites in its promoter for NFκB and AP-1 (Mukaida *et al,* 1994; Kunsch and Rosen 1993; Roebuck 1999). The present study has shown that WIN-55 decreased IL-1β induced IκB phosphorylation (Chapter 4), therefore preventing the translocation of NFκB to the nucleus to induce target genes. In addition WIN-55 reduced IL-1β induced c-Jun phosphorylation (Chapter 4), which is required for the activity of AP-1 transcription factor (Karin *et al,* 1997). Data presented here suggests a possible mechanism via which WIN-55 may act to prevent IL-1β induced IL-8 expression via inhibition of its transcriptional regulation by NFκB and AP-1. Interestingly in RA synovial fibroblasts, IL-1β induced activation of IL-8 was shown to be regulated by NFκB but not c-Jun (Georganas *et al,* 2000).

3.6.2 NGF

WIN-55 has been shown to have analgesic activities and reduce nociception in animal models of inflammatory pain (Ebrahimzadeh and Haghparast 2011; Burgos *et al,* 2010). In addition other cannabinoid agonists have been shown to have antinociceptive effects in animal models of arthritis (Schuelert and McDougall 2011; Smith *et al,* 1998; Cox and Welch 2004; Cox *et al,* 2007; Schuelert and McDougall 2008). This study has shown that IL-1β induced both NGF and substance P mRNA expression; conversely WIN-55 both alone and in combination with IL-1β produced unexpected findings. WIN-55 reduced IL-1β induced NGF mRNA expression however expression levels remained above basal levels. Interestingly, WIN-55 treatment alone increased NGF expression above basal levels.

NGF is thought to contribute to the inflammatory process during RA, as NGF secreted by fibroblast like synovial cells promotes the survival of activated autocrine T cells and the proliferation of synovial cells, a process which is also thought to occur in OA synovium (Raychaudhuri *et al,* 2011). Signalling pathways initiated by NGF include MAPKs and NFκB pathways, which activate genes involved in neurite outgrowth, neuronal differentiation and neuronal survival (Reichardt 2006). Increases in NGF have been associated with increased levels of IL-1β as shown in animal models of arthritis (Manni and Aloe 1998). Furthermore, treatment of human synovial fibroblasts with IL-1β increased NGF levels, however in the same study NGF was shown to reduce IL-1β induced expression of TNF and iNOS, concluding that NGF may be involved in modulating the inflammatory response in joints (Manni *et al,* 2003). In the present study it was shown that WIN-55 reduced IL-1β induced mRNA expression of NGF but these levels remained above basal levels. WIN-55 treatment alone also increased NGF mRNA expression. In contrast, endogenous cannabinoid PEA inhibited the release of NGF from human mast cells (Cantarella *et al,* 2011), suggesting that distinct cannabinoid ligands or cell types may induce differential effects on NGF expression.

Other studies have found that NGF may possess anti-inflammatory actions as blocking endogenous NGF in animal models induced joint inflammation (Manni *et al,* 2002). Moreover it has been postulated that NGF may have protective properties in human OA chondrocytes (Iannone *et al,* 2002). Chondrocytes

obtained from higher grades of degenerative cartilage have increased levels of NGF expression compared to chondrocytes obtained from non-degenerative cartilage, it was therefore proposed that NGF stimulates chondrocyte metabolism and promotes cartilage repair (Iannone *et al,* 2002). These findings suggest that NGF produced by chondrocytes may be involved in processes that differ from those of NGF found at the site of innervation at the subchondral junction which is thought to be associated with pain in OA (Walsh *et al,* 2010). To identify the role of NGF in regulating chondrocyte metabolism in addition to the effects of WIN-55 on its expression requires further investigation.

3.6.3 Substance P

Substance P plays an important role in pain signalling and has proinflammatory effects in arthritis (Keeble and Brain 2004). Substance P is released along with glutamine after nociceptive fibres are stimulated (Keeble and Brain 2004). Nerve fibres positive for substance P have been shown to be present in cells lining the synovium with evidence of some nerve fibres branching towards the joint (Iwasaki *et al,* 1995). Infusion of substance P into the knee joint increases the severity of arthritis and endogenous substance P is elevated in the synovial fluid of OA and RA patients (Levine *et al,* 1984; Im *et al,* 2008). Furthermore, in OA chondrocytes expression of substance P and its receptor NK-1 are increased suggesting that it may play a role in the pathogenesis of arthritis (Im *et al,* 2008).

A possible mechanism via which cannabinoids are thought to have analgesic effects is via inhibition of substance P release from primary afferent terminals (Zhang *et al,* 2010). In an *in vivo* study on mouse spinal cord, CB1 antagonist SR141716A increased capsaicin induced substance P release and endogenous cannabinoid AEA inhibited these effects suggesting that CB1 plays a role in inhibiting substance P release (Lever and Malcangio 2002). Conversely at high concentrations AEA has been shown to induce the release of substance P via activation of vanilloid VR1 receptor (Tognetto *et al,* 2001), suggesting that AEA antinociceptive actions at cannabinoid receptors are concentration dependent. Endocannabinoids in particular AEA, are readily hydrolysed by FAAH and inhibition of this enzyme in animal models of osteoarthritis was shown to reduce nociception (Schuelert *et al,* 2011). In contrast, FAAH1 inhibitor PF-04457845 was trialed in patients with knee OA, and whilst the inhibitor increased

endogenous cannabinoid AEA and related FAA, PEA, OEA and linoleoylethanolamide (LEA), it failed to produce analgesia (Huggins *et al,* 2012).

This present study showed that IL-1β induced the mRNA expression of substance P in human chondrocytes, an observation also made by Im *et al* (2008). The effects of WIN-55 on the release of substance P from chondrocytes were also determined and showed that WIN-55 both alone and in combination with IL-1β induced mRNA expression of substance P.

Other studies that have investigated the effects of substance P on chondrocytes, have shown that it may not act as a classic neuropeptide but may have distinct chondroprotective activities (Opolka *et al,* 2012). Intracellular levels of substance P are increased in both OA lesions and in active proliferating human fetal chondrocytes, suggesting that substance P is involved in the stimulation of chondrocyte proliferation (Iannone and Lapadula 1998). Whilst having no effect on ECM production, substance P increased chondrocyte proliferation and cell adhesion contacts via its receptor NK-1 (Opolka *et al,* 2012). In addition another neurotransmitter, norepinephrine, decreased the apoptosis rate of chondrocytes (Opolka *et al,* 2012). Conversely, (Im *et al,* 2008) showed that in human chondrocytes, substance P increases protein secretion of MMP-13 via the ERK1/2 and NFκB pathways, in addition to decreasing proteoglycan production, suggesting catabolic actions of this peptide in articular cartilage. In addition other functions of substance P have also been identified, it has also been suggested that substance P plays a role in the response of chondrocytes to mechanical stimulation via the NK-1 receptor (Millward-Sadler *et al,* 2003). Together these studies suggest that in chondrocytes, substance P plays a role independent of pain signalling and has a potential role in chondrocyte metabolism and cell function. To determine the effects of substance P in response to WIN-55 and or IL-1β stimulation in human chondrocytes requires further investigation.

3.7 Summary

The study has demonstrated that WIN-55 decreased IL-1β induced IL-8 mRNA expression, suggesting that cannabinoids display anti-inflammatory properties during OA. Furthermore, WIN-55 also reduced IL-1β induced NGF mRNA

expression, however conversely WIN-55 treatment alone significantly induced an increase in basal levels of NGF. In addition, WIN-55 alone and in combination with IL-1β induces the gene expression of substance P. The significance of this requires further investigation since differential roles of NGF and substance P, independent of pain signalling in chondrocytes, has been demonstrated.

4 The Effects of WIN-55 on IL-1β Signalling Pathways

4.1 Introduction

There is increasing evidence to suggest that IL-1β plays a role in the initiation and subsequent progression of OA, making it a key therapeutic target. IL-1β is induced during OA and is synthesised by chondrocytes, cells of the synovium and subchondral bone (Kapoor *et al,* 2011). Increases in IL-1β results in an increase in MMPs and a decrease in the synthesis of cartilage ECM macromolecules via the inhibition of anabolic metabolism by chondrocytes (Goldring *et al,* 1994; Mengshol *et al,* 2000; Mengshol *et al,* 2001; Goldring 1996).

Inflammation is evident in both the early and late stages of OA, and is predominantly driven by IL-1β and TNF with the involvement of other proinflammatory cytokines including IL-6 and chemokines such as IL-8 (Kapoor *et al,* 2011). Reduction of IL-1β expression in chondrocytes using RNA interference resulted in a decrease in mRNA levels of inflammation related genes including cytokines, chemokine IL-8, MMP-3, MMP-2, IFNγ and iNOS, suggesting that knockdown of IL-1β may be chondroprotective (Santangelo and Bertone 2011). Conversely IL-1β gene knockdown in a mouse model of OA showed accelerated development of lesions, these findings suggest that IL-1β also plays an important role in maintaining cartilage ECM turnover and homeostasis under normal physiological conditions (Clements *et al,* 2003).

4.1.1 IL-1β Signalling Pathways in OA

IL-1β initiates its effects via binding to its receptor IL-1RI, a cell surface receptor expressed by chondrocytes and synovial fibroblasts (Martel-Pelletier *et al,* 1992; Sadouk *et al,* 1995). During OA, IL-1RI expression is increased on human chondrocytes and synovial fibroblasts making these cells more responsive to IL-1β stimulation (Martel-Pelletier *et al,* 1992; Sadouk *et al,* 1995). Consequently less IL-1β is required to induce MMP secretion in OA chondrocytes compared to normal chondrocytes (Martel-Pelletier *et al,* 1992).

Signalling pathways direct an extracellular signal from the cell membrane to the cell nucleus. Ligands bind to receptors expressed on the cell surface, which induces interaction of the receptors intracellular domain with intracellular signalling proteins (Cooper 2000). These interactions induce a cascade of protein interactions and phosphorylation events, subsequently leading to the

translocation of transcription factors to the nucleus (Cooper 2000). Transcription factors recognise and bind to specific DNA-binding elements in the promoter regions of target genes either inducing or suppressing the expression of mRNA (Handel and Girgis 2001).

WIN-55 inhibits the IL-1β induced mRNA and protein expression of MMP-3 and -13 and mRNA expression of their inhibitors TIMP-1 and -2 (Chapter 2, Dunn *et al,* 2013). It is important to elucidate a possible mechanism via which this may occur to enable the potential use of cannabinoids, possibly based on WIN-55, in OA therapies to be assessed. During OA IL-1β induces a cascade of intracellular signalling pathways, which initiate the expression of matrix degrading enzymes (Mengshol *et al,* 2000). c-Jun, p38, ERK1/2 and NFκB are activated by IL-1β stimulation in both normal and OA cartilage particularly in the upper zones of the cartilage (Fan *et al,* 2007). In addition, IL-1β stimulation was shown to reduce collagen type II expression via p38 MAPK activation in human chondrocytes (Robbins *et al,* 2000).

During OA IL-1β induces the phosphorylation, ubiquitination and subsequent degradation of inhibitory protein (IκB) allowing NFκB to translocate to the nucleus of chondrocytes, where it regulates the expression of other proinflammatory mediators such as iNOS, COX-2 and matrix degrading enzymes MMP-1, MMP-9, MMP-13 and ADAMTS-4 (Hayden and Ghosh 2008; Roman-Blas and Jimenez 2006). As well as orchestrating multiple inflammatory responses in OA, NFκB is involved in the differentiation of chondrocytes to a more hypertrophic like phenotype (Marcu *et al,* 2010). Thus targeting the NFκB signalling pathway has been suggested as a therapeutic strategy in the treatment of OA and RA (Marcu *et al,* 2010; Roman-Blas and Jimenez 2006). Pharmacological inhibitors of the NFκB signalling pathway have been shown to have protective properties in animal models of RA (Marcu *et al,* 2010). However NFκB or MAPK inhibition has received little attention in OA models (Kapoor *et al,* 2011; Marcu *et al,* 2010; Saklatvala 2007).

4.2 Aims and Objectives:

Aim: To determine the effects of WIN-55 on IL-1β signalling pathways MAPKs and NFκB in human OA chondrocytes

Objectives:

- To determine the effects of WIN-55 on IL-1β induced phosphorylation of NFκB using immunocytochemistry
- To determine the time dependent effects of WIN-55 on the classical IL-1β signalling MAPK, ERK1/ERK2.
- To determine the effects of WIN-55 on ERK1/ERK2, IκB, p38 and c-Jun phosphorylation in the presence of IL-1β using cell based ELISA.
- To determine the effects of WIN-55 on the phosphorylation of 46 different protein kinases in the presence of IL-1β using a proteome array.

4.2.1 Experimental design

The effects of WIN-55 on IL-1β induced signalling pathways in chondrocytes obtained from OA cartilage were investigated. Cartilage tissue was graded macroscopically 0-4 using the Outerbridge classification (Cameron *et al,* 2003). Chondrocytes were isolated from grade 2 or 3 cartilage tissue as representative of low degenerate and intermediate degenerate cartilage tissue. Cartilage from grade 4, severe degenerate tissue, was not used in the study, as the cell yield obtained was not sufficient. Chondrocytes were cultured in monolayer and cells were stimulated with IL-1β to induce catabolic responses. Chondrocytes were expanded in monolayer to passage 2 and stimulated with 10 ng/ml IL-1β to induce intracellular signalling cascades. Chondrocytes were pre-treated or co-treated with 10 μM WIN-55 with and without 10 ng/ml IL-1β. Immunocytochemistry was used to investigate the phosphorylation of NFκB. Cell based ELISA was used to measure the phosphorylation of cell signalling molecules p38, ERK1/ERK2, IκB and c-Jun and the phosphorylation of 46 different kinases was investigated using a protein array.

4.3 Methodology

4.3.1 Human OA Cartilage Samples

Primary Human chondrocytes were obtained from articular cartilage removed from patients with symptomatic OA at the time of total knee replacement as described in section 2.4.1.

4.3.2 Macroscopic Grading of Cartilage Tissue

Cartilage tissue was macroscopically graded 0-4 using the Outerbridge classification at time of surgery prior to isolation of chondrocytes (Cameron *et al,* 2003) as described in section 2.4.2.

4.3.3 Isolation of Human Chondrocytes

Human chondrocytes were isolated from cartilage as described in section 2.4.3.

4.3.4 OA Patient Samples

Chondrocytes cultures were derived from OA patient samples of macroscopic grades 2 or 3; HC3(3), HC3(4), HC5(1) HC11(3), HC15(4), HC16(4), HC21(4), HC22(4) and HC23(4) (Table 4.1). Full patient details are shown in Appendix 1.

Analysis Performed	**Monolayer**	
	Grade 2	**Grade 3**
NFκB Immunocytochemistry	HC3(3)	Not investigated
Cell based ELISA (ERK1/ERK2, c-Jun, p38, IκB Phosphorylation)	HC3(4), HC5(1), HC23(4), HC21(4)*	Not investigated
Protein array	Not investigated	HC22(4)

Table 4.1 The patient samples used for each analysis performed on chondrocytes obtained from different macroscopic grades of OA cartilage. Full details of samples used in these investigations can be found in Appendix 1. *Indicates the patient sample used for the time course investigation of ERK1/ERK2 phosphorylation following WIN-55 treatment (section 4.3.9.1).

4.3.5 Culture of OA Chondrocytes for NFκB Immunocytochemistry

Chondrocytes were cultured in monolayer until 80% confluent at passage 2. Following trypsinisation, as outlined in section 2.4.5, cells were centrifuged at 400g for 10 minutes and resuspended in complete media. Cells were counted using trypan blue exclusion using the Countess cell counter. Chondrocytes were seeded at $1x10^4$ cells per well in 8 well chamber slides in complete media. Cells were allowed to adhere overnight at 37°C in a humidified atmosphere of 5% CO_2 prior to IL-1β and WIN-55 treatment.

4.3.6 WIN-55 and IL-1β Treatment of OA Chondrocytes for NFκB Immunocytochemistry

Cells were washed twice in 1xPBS and complete media replaced with 200 µl serum free media+BSA per well supplemented with 10 µM WIN-55 for 1 hour both with and without 10 ng/ml IL-1β for the last 30 minutes of stimulation and incubated at 37°C. DMSO (0.1%) was used as a vehicle control at the same concentration present in 10 µM WIN-55. Untreated cells were used as control.

4.3.7 NFκB Immunocytochemistry

Culture media was removed and cells washed twice in 1xPBS. Chambers were removed from slides and cells were fixed and permeablised in ice-cold methanol at -20°C for 4 minutes followed by incubation in ice-cold acetone at -20°C for 2 minutes. Cells were washed in 1xTris buffered saline (TBS; Fisher Scientific) for 5 minutes. Non-specific binding sites were blocked with 25% v/v goat serum (Abcam) in 1% BSA in 1xTBS for 1 hour at room temperature. Cells were incubated with rabbit polyclonal antibody (Abcam) against p65 NFκB (1/50) overnight at 4°C in a humidified chamber. For negative controls, cells were incubated without primary antibody. Cells were washed 3 times in 0.1% Tween 20 in 1xTBS for 5 minutes each to remove unbound primary antibody. Following washing cells were incubated with FITC conjugated goat anti-rabbit IgG (1/250) (Abcam) at room temperature for 30 minutes. Cells were washed 3 times in 0.1% Tween 20 in 1xTBS for 5 minutes each to remove unbound secondary antibody. Slides were mounted with Vectorshield hardset mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories).

4.3.8 Immunocytochemistry Visualisation

Cells were visualised and images captured on the Zeiss laser scanning confocal microscope at a magnification of 630X magnification using the Zen 2009 operating system. Due to limitations of the confocal microscope DAPI stain could not be visualised. The percentage of positive cells was determined by counting 200 cells per well. Positive staining for NFκB was observed in both the cytoplasm and nucleus, thus a separate percentage for each cellular localisation was determined (Figure 3.2A).

4.3.9 Cell Based ELISA on Cultured OA Chondrocytes.

Chondrocytes were cultured in monolayer until 80% confluence at passage 2. Following trypsinisation, as outlined in section 2.4.5, cells were centrifuged at 400g for 10 minutes and resuspended in complete media. Cells were counted using trypan blue exclusion on the Countess cell counter (Invitrogen). Chondrocytes were seeded at $1x10^5$ cells per well in a black 96 well microplates (R&D Systems) in complete media. Cells were allowed to adhere overnight at 37°C in a humidified atmosphere of 5% CO_2 prior to IL-1β and WIN-55 treatment.

4.3.9.1 Cell Based ELISA ERK1/ERK2 WIN-55 and IL-1β Time Course Treatment

Cells were washed twice in 1xPBS and the complete media replaced with 200 µl serum free media+BSA. Cells were treated with 10 ng/ml IL-1β for 30 minutes or 1 hour. Cells were treated with 10 µM WIN-55 for 30 minutes, 1, 3, 6, 24 or 48 hours alone and in combination with 10 ng/ml IL-1β for the last 30 minutes of the WIN-55 treatment. 0.1% DMSO was used as a vehicle control at the same concentration present in 10 µM WIN-55 and cells were treated for 30 minutes, 1, 3, 6, 24 or 48 hours. Untreated cells in culture for 48 hours were used as control.

4.3.9.2 Cell Based ELISA optimised WIN-55 and IL-1β treatments of OA Chondrocytes

Cells were washed twice in 1xPBS and the complete media replaced with 200 µl serum free media+BSA per well supplemented with 10 µM WIN-55 for 48 hours both with and without 10 ng/ml IL-1β for the last 30 minutes of the stimulation. 0.1% DMSO was used as a vehicle control at the same

concentration present in 10 µM WIN-55. Untreated cells in culture for 48 hours were used as control.

4.3.9.3 Cell Based ELISA Principle

The Cell Based ELISA allows for the fluorogenic detection of phosphorylated ERK1/ERK2, c-Jun, IκB and p38 in whole cells (Figure 4.1). Cells are cultured in 96-well plates and stimulated with ligands. Following stimulation cells are fixed and permeabilsed. The assay involves the use of double immunoenzymatic labels to measure protein phosphorylation. The cells are simultaneously incubated with two primary antibodies for phosphorylated and total protein (Table 4.2). The two primary antibodies recognising the different primary antibody species are labelled with HRP or alkaline phosphatase (AP). Two spectrally different fluorogenic substrates are used for detection of HRP or AP.

4.3.10 Cell Based ELISA

4.3.10.1 Fixing and Blocking Cells

Following treatment as outlined in section and 4.3.9.1 and 4.3.9.2 culture media was removed and the cells were fixed in 4% formalin v/v in 1xPBS. The amount of phosphorylated c-Jun, IκB, p38 or ERK1/ERK2 was measured using a cell based ELISA (R&D systems, UK). The formalin was removed and each well washed 3 times with 200 µl 1x wash buffer for 5 minutes each on an orbital shaker. The wash buffer was removed and 100 µl of quenching buffer (0.6% hydrogen peroxide (H_2O_2)) was added to each well and incubated at room temperature for 20 minutes. The quenching buffer was removed and each well washed 3 times with 200 µl 1x wash buffer for 5 minutes each on an orbital shaker. The wash buffer was removed and 100 µl of blocking buffer (10%FBS) was added to each well and incubated at room temperature for 1 hour. The blocking buffer was removed and the cells washed 3 times with 200 µl 1x wash buffer for 5 minutes each on an orbital shaker.

4.3.10.2 Binding of Primary and Secondary Antibodies

The wash buffer was removed and primary antibodies were diluted 1:100 in blocking buffer immediately prior to use (Table 4.2) and 100 µl was added to each well and incubated for 16 hours at 4°C. Cells were incubated with blocking buffer alone which served as the negative control. The primary antibody was

removed and the cells washed 3 times with 200 µl 1x wash buffer for 5 minutes each on an obital shaker. HRP-conjugated IgG and AP-conjugated IgG secondary antibodies (Table 4.2) were diluted 1:100 in blocking buffer immediately before use and 100 µl was added to each well including the negative control wells and incubated at room temperature for 2 hours.

4.3.10.3 Fluorogenic Detection

The secondary antibody was removed and the cells washed 2 times with 200 µl 1x wash buffer and 2 times with 200 µl 1xPBS for 5 minutes each on an orbital shaker. The wash buffer was removed and 75 µl of Substrate F1 (substrate for HRP) was added to each well. Following incubation with Substrate F1 for 60 minutes at room temperature protected from light, 75 µl of Subtrate F2 (substrate for AP) was added to each well and incubated at room temperature for a further 20 minutes protected from light. The plate was read fluorometrically using the Tecan Infinite 200 Pro with excitation at 540 nm and emission at 600 nm, then with excitation at 360 nm and emission at 450 nm. The readings at 600 nm represented the amount of phosphorylated protein and the readings at 450 nm represented the amount of total protein regardless of its phosphorylation status.

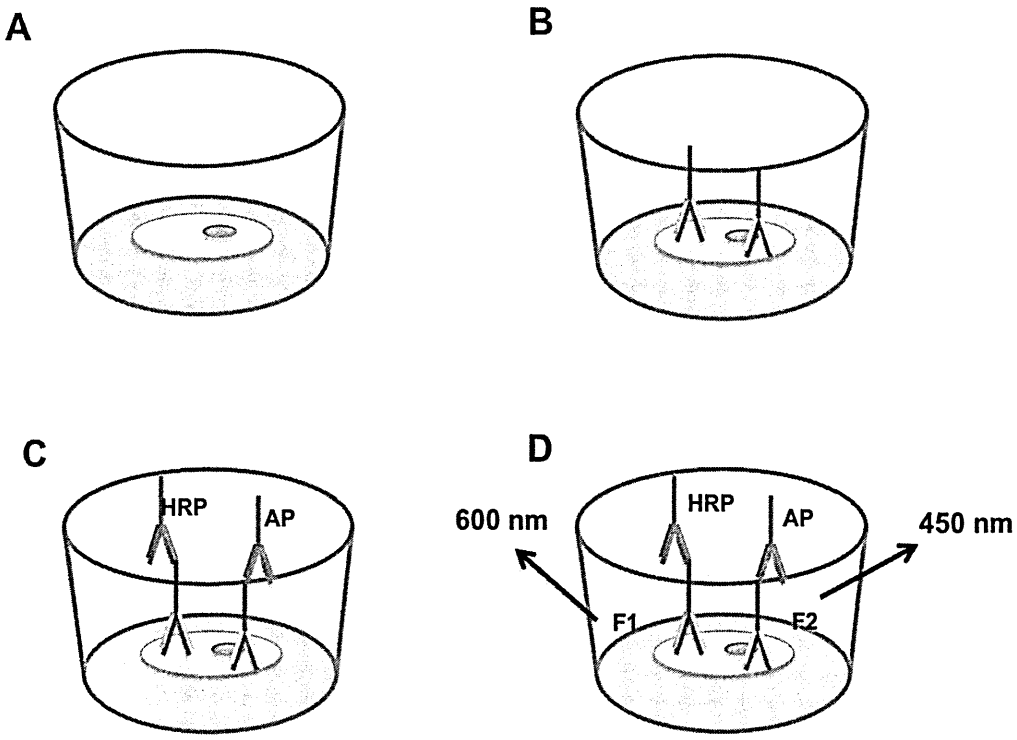

Figure 4.1 The Principle of Cell Based ELISA. (A) Cells are seeded in a 96-well plate and stimulated with ligands (WIN-55 +/- IL-1β). Cells are fixed, permeablised and blocked. **(B)** Primary antibodies are added and incubated overnight at 4°C. **(C)** Secondary antibodies are incubated with primary antibodies conjugated to HRP and AP. **(D)** Fluorogenic substrate F1 and F2 are added and the fluorescence measured.

Primary Antibody	Secondary Antibody	Phosphorylation Site Detected
Rabbit anti-phospho-ERK1/ERK2 (Phosphorylated) Mouse anti-ERK1/ERK2 (Total)	HRP-conjugated goat anti-rabbit IgG AP-conjugated goat-anti-mouse IgG	ERK1-T202/Y204 ERK2-T185/Y187
Mouse anti-phospho-IκB-α (Phosphorylated) Rabbit anti-GAPDH (Total)	HRP-conjugated donkey anti-mouse IgG AP-conjugated donkey anti-rabbit IgG	S32/S36
Rabbit anti-phospho-p38 (Phosphorylated) Mouse anti-p38 (Total)	HRP-conjugated goat anti-rabbit IgG AP-conjugated goat anti mouse IgG	T180/Y182
Rabbit anti-phospho-c-Jun (Phosphorylated) Mouse anti-c-Jun (Total)	HRP-conjugated goat anti-rabbit IgG AP-conjugated goat anti-mouse IgG	S63

Table 4.2 The primary and secondary antibodies used to detect phosphorylated ERK1/ERK2, IκB, p38 and c-Jun and a normalization antibody that recognises the total protein regardless of phosphorylation status

4.3.11 Principle of the Protein Array

The human Phospho-Kinase Array (R&D Systems) allows for the simultaneous detection of relative levels of phosphorylation of 46 kinase phosphorylation sites. Nitrocellulose membranes are spotted with capture and control antibodies in duplicate. Cell lysates are incubated with the membranes to allow the binding of target proteins. Membranes are incubated with a cocktail of biotinylated detection antibodies. Streptavidin-HRP and chemiluminescent detection reagent are used to detect the signal produced at each capture spot corresponding to the amount of phosphorylated protein bound.

4.3.11.1 Protein Array WIN-55 and IL-1β Treatments of OA Chondrocytes

Chondrocytes were cultured in monolayer until 80% confluent at passage 2 in T75 culture flasks as described in section 2.4.5. Cells were washed twice in 1xPBS and the complete media replaced with 10 ml serum free media+BSA supplemented with 10 µM WIN-55 and incubated for 48 hours both with and without 10 ng/ml IL-1β for the last 30 minutes of stimulation. DMSO (0.1%) was used as a vehicle control at the same concentration present in 10 µM WIN-55.

4.3.11.2 Protein Extraction

Following treatment of cells as outlined in section 4.3.11.1 cells were washed twice in 1xPBS and incubated with 500 µl cell lysis buffer (R&D systems) with 10% protease inhibitor cocktail (Sigma-Aldrich) for 30 minutes at 4°C with gentle agitation. Samples were centrifuged at 14,000g for 5 minutes and the supernatant transferred to a clean Eppendorf. Protein concentration was determined using the Bio-rad protein Assay (Bio-rad). Briefly protein standards of 1.44, 0.72, 0.36, 0.18, 0.09, 0.045, 0.025 and 0.01125 mg/ml were produced from a stock of 1.44 mg/ml BSA. 5 µl of sample, standard or lysis buffer (negative control) was added to a 96 well plate, 250 µl of protein assay dye diluted 1/5 in deionised H_2O was added to each well and the absorbance read at 570 nm using the Wallac Victor 1820 plate reader. The amount of protein in each sample was determined using the standard curve and linear regression analysis (y=mx+c where y=absorbance; m=gradient; x=protein concentration; c= y intercept).

4.3.11.3 Protein Array

The protein Array was used as per the manufacturer's instructions (R&D Systems). Briefly, 1ml of Array Buffer 1 was added to each membrane (membrane A and B each containing different capture antibodies) and incubated at room temperature for one hour on a flat bed shaker. Cell lysates were diluted 1:5 with Array Buffer 1 to give a final concentration of 55 µg of protein per membrane. Array Buffer 1 was removed from each membrane and 1 ml of prepared sample added to both of the corresponding part A and part B membrane and incubated overnight at 4°C on a flat bed shaker. Membranes were washed for 10 minutes in 1xWash Buffer for a total of three washes. Detection Antibody Cocktail A and B were reconstituted in 100 µl deionised water. Following reconstitution 20 µl of detection antibody cocktail A was diluted in 1 ml of 1xArray Buffer 2/3 and 1 ml of the diluted detection antibody cocktail was added to membrane A and 20 µl of detection antibody cocktail B was diluted in 1 ml of 1xArray Buffer 2/3 and 1 ml of the diluted detection antibody cocktail was added to membrane B. Detection antibody cocktails were incubated with their appropriate membranes at room temperature for 2 hours on a flat bed shaker. Membrane part A and part B were washed separately in 1xWash Buffer for 10 minutes for a total of three washes. Streptavidin-HRP was diluted 1/2000 in 1xArray Buffer 2/3 and 1 ml was added to each well with membranes A and B and incubated for 30 minutes at room temperature on a orbital shaker. Both membranes were washed in 1xWash Buffer for 10 minutes for a total of three washes. Membranes were incubated with 1 ml of chemiluminescent reagent for detection of phosphorylated target proteins for 5 minutes.

4.3.11.4 Protein Array Analysis

The chemiluminescent signals from the membrane were detected using the UVP transilluminatior and Labworks version 3.0 image analysis software. Multiple exposure times were applied and the pixel dot intensities on each membrane along with background values were exported to Excel. The average signal of the pair of duplicate spots, representing each phosphorylation kinase protein was determined along with the averages for the positive controls. The averaged background signal from each spot was subtracted from each of the signal spots. Signal spots were normalised to the positive control and then further normalised to the DMSO control and data expressed as relative protein expression.

4.3.12 Statistical Analysis

Statistical analysis was performed as outlined in section 2.5 for cell based ELISA and protein array analysis. Statistical analysis could not be performed on immunocytochemistry data as only one repeat from one patient sample was investigated.

4.4 Results

4.4.1 The Effects of WIN-55 on IL-1β Induced NFκB Phosphorylation

Following IL-1β stimulation for 30 minutes there was an 88% increase in positive staining for nuclear NFκB compared to DMSO control indicated by the blue arrow (Figure 4.2B(c)). Following 1 hour pre-treatment with WIN-55 prior to 30 minute IL-1β stimulation there was an 81% increase in positive staining for nuclear NFκB compared to DMSO control as indicated by the blue arrow (Figure 4.2B (d)), in addition there was cytoplasmic staining observed as indicated by the red arrow (Figure 4.2B (d)). WIN-55 treatment alone resulted in 77% of cells staining positively for cytoplasmic NFκB compared to DMSO control (Figure 4.2B(e)).

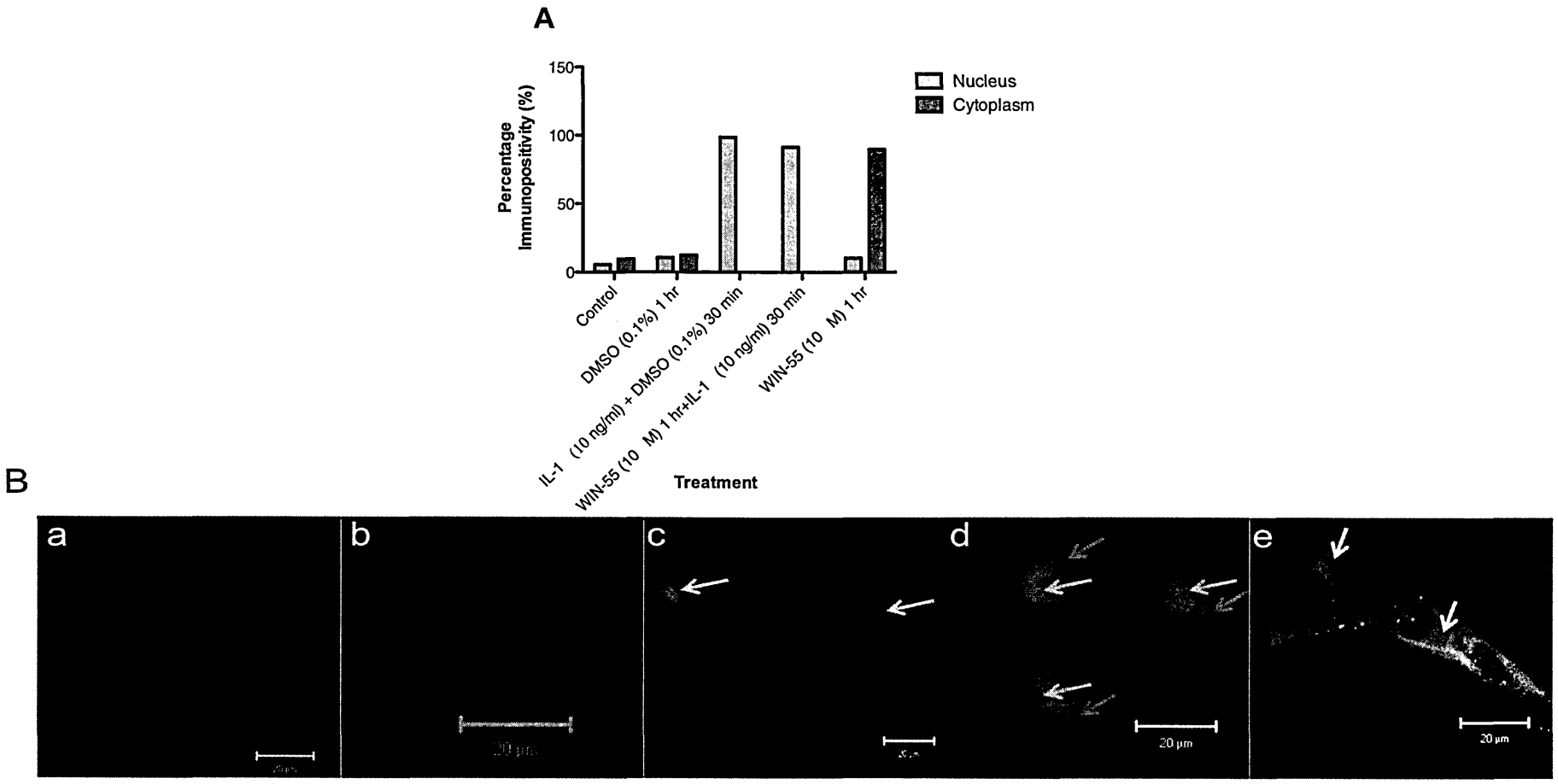

Figure 4.2 The effects of WIN-55 on IL-1β induced NFκB phosphorylation. (A) Untreated Control **(B)** DMSO control **(C)** IL-1β and DMSO **(D)** WIN-55 and IL-1β **(E)** WIN-55. Following IL-1β stimulation for 30 minutes both alone and in combination with WIN-55 for 1 hour induces an increase in positive staining for nuclear NFκB (blue arrows). Cytoplasmic staining following WIN-55 treatment for 1 hour in combination with IL-1β for 30 minutes as indicated by the red arrows was observed. WIN-55 treatment alone for 1 hour resulted in an increase in positive staining for cytoplasmic NFκB (yellow arrows). Data represents the percentage immunopositivity. n=1 obtained from one patient sample HC3(3).

4.4.2 The Time-dependent Effects of WIN-55 on IL-1β Induced ERK/1ERK2 Phosphorylation.

The effects of WIN-55 on IL-1β induced ERK1/ERK2 phosphorylation was investigated using cell-based ELISA. Since a pre-treatment of WIN-55 for 1 hour had no effect on IL-1β induced phosphorylation of NFκB (section 4.4.1) a time-course of WIN-55 treatments was investigated. DMSO treatment alone at 0.1% for 30 minutes, 1, 3, 6 and 24 hours had no effect on ERK1/ERK2 phosphorylation compared to untreated control (Data not shown). ERK1/ERK2 phosphorylation was significantly induced by IL-1β stimulation for 10 or 30 minutes ($p<0.001$ and $p<0.01$) but not following 1 hour IL-1β stimulation compared to the appropriate DMSO control of 10 or 30 minutes and 1 hour respectively (Figure 4.3). WIN-55 treatment for 30 minutes in combination with IL-1β stimulation for 30 minutes did not counteract the effects on ERK1/ERK2 phosphorylation compared to IL-1β stimulation alone for 30 minutes (Figure 4.3). Pre-treatment of chondrocytes with WIN-55 for 1, 3, 6 and 24 hours prior to 30 minute IL-1β stimulation did not reduce ERK1/ERK2 phosphorylation compared to IL-1β stimulation alone for 30 minutes (Figure 4.3). However WIN-55 pre-treatment for 48 hours significantly reduced IL-1β induced phosphorylation to basal levels compared to IL-1β stimulation alone for 30 minutes ($p<0.01$) (Figure 4.3). ERK1/ERK2 phosphorylation remained at basal levels following WIN-55 treatment for 30 minutes, 1, 3, 6 and 24 hours. WIN-55 treatment alone for 48 hours significantly reduced ERK1/ERK2 phosphorylation below basal levels compared to DMSO treatment alone for 48 hours ($p<0.05$) (Figure 4.3).

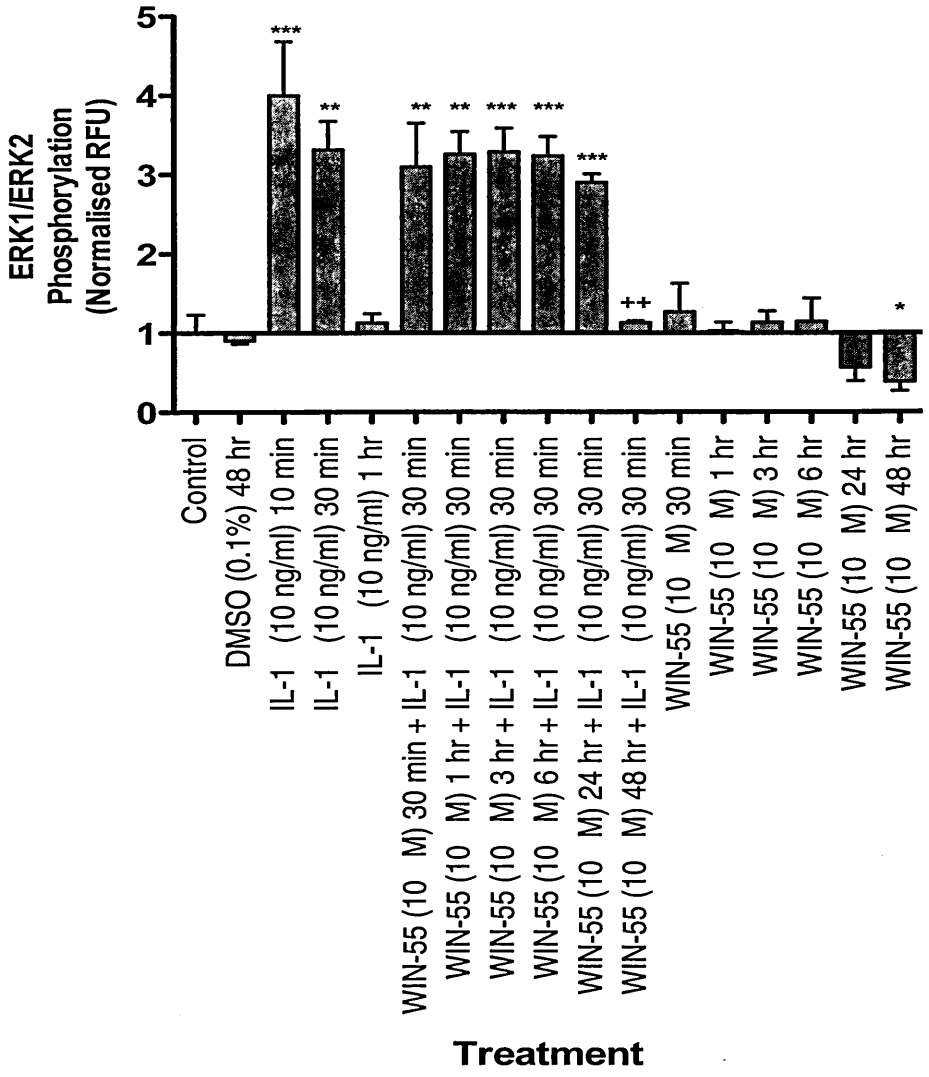

Figure 4.3 The effects of WIN-55 on IL-1β induced ERK1/ERK2 phosphorylation over time. IL-1β stimulation for 10 or 30 minutes but not 1 hour significantly induced ERK1/ERK2 phosphorylation. WIN-55 pre-treatment for 48 hours significantly reduced IL-1β induced ERK1/ERK2 phosphorylation. WIN-55 treatment for 48 hours significantly reduced ERK1/ERK2 phosphorylation below basal levels compared to DMSO control. Data represents mean fold change of phosphorylation normalised to internal total unphosphorylated protein and untreated control ± SEM.*$p<0.05$, **$p<0.01$ ***$p<0.001$ compared to DMSO control, ++$p<0.01$ compared to IL-1β stimulation for 30 minutes. n=3 obtained from one patient sample (HC21(4)).

4.4.3 The Effects of WIN-55 on IL-1β Induced Signalling Pathways

As 48-hour pre-treatment was determined to be the time point at which WIN-55 reduced IL-1β induced ERK1/ERK2 phosphorylation, a 48 hour pre-treatment was used to investigate the effects of WIN-55 on IL-1β induced ERK1/ERK2, IκB, c-Jun and p38 phosphorylation on further patient samples using the cell based ELISAs.

4.4.3.1 ERK1/ERK2

IL-1β stimulation for 30 minutes significantly increased ERK1/ERK2 phosphorylation compared to DMSO control ($p<0.001$) (Figure 4.4). WIN-55 pre-treatment in combination with IL-1β for the last 30 minutes significantly reduced ERK1/ERK2 phosphorylation below basal levels compared to DMSO control and IL-1β stimulation alone ($p<0.001$) (Figure 4.4). WIN-55 treatment alone for 48 hours significantly reduced ERK1/ERK2 phosphorylation below basal levels compared to DMSO alone ($p<0.001$) (Figure 4.4).

4.4.3.2 IκB

IL-1β stimulation for 30 minutes significantly increased IκB phosphorylation compared to DMSO control ($p<0.05$) (Figure 4.5). WIN-55 pre-treatment in combination with IL-1β for the last 30 minutes significantly reduced IκB phosphorylation below basal levels compared to DMSO control and IL-1β stimulation alone ($p<0.001$) (Figure 4.5). Phosphorylation of IκB was reduced below basal levels compared to DMSO control following WIN-55 treatment for 48 hours ($p<0.001$) (Figure 4.5).

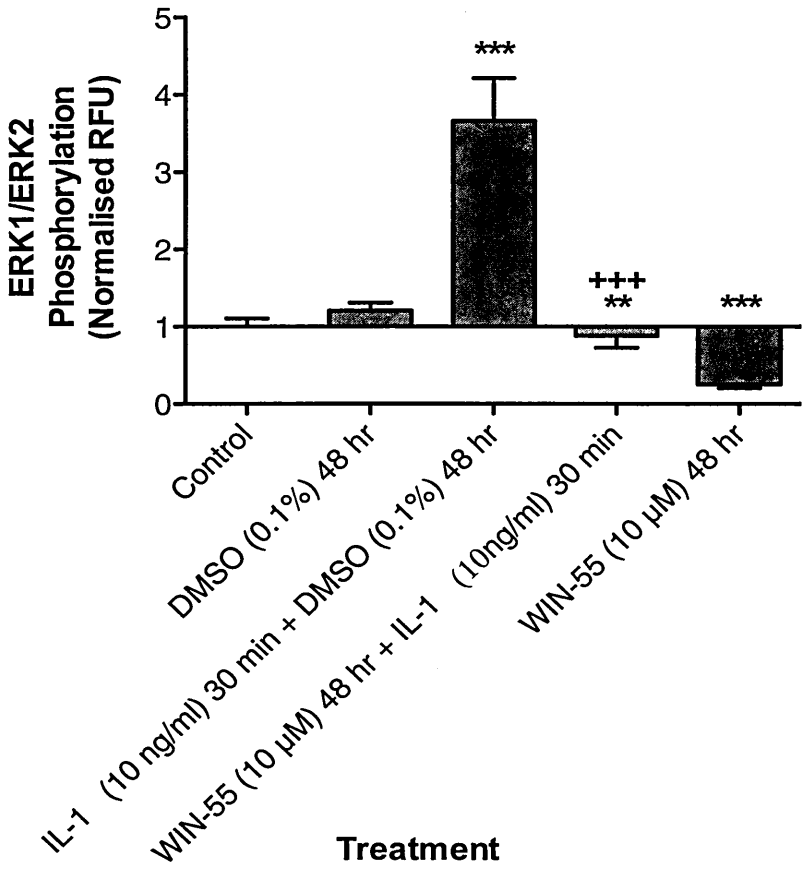

Figure 4.4 The effects of WIN-55 on IL-1β induced ERK1/ERK2 phosphorylation. IL-1β stimulation for 30 minutes induced phosphorylation of ERK/ERK2. WIN-55 pre-treatment for 48 hours in combination with IL-1β stimulation for the last 30 minutes significantly reduced phosphorylation of ERK1/ERK2 compared to 30 minute IL-1β stimulation alone. WIN-55 treatment alone for 48 hours significantly reduced ERK1/ERK2 phosphorylation below basal levels. Data represents mean fold change of phosphorylation normalised to internal total unphosphorylated protein and untreated control ± SEM.**$p<0.01$, ***$p<0.001$ compared to DMSO control and +++$p<0.001$ compared to IL-1β alone. n=9 obtained from 3 macroscopic grade 3 patients.

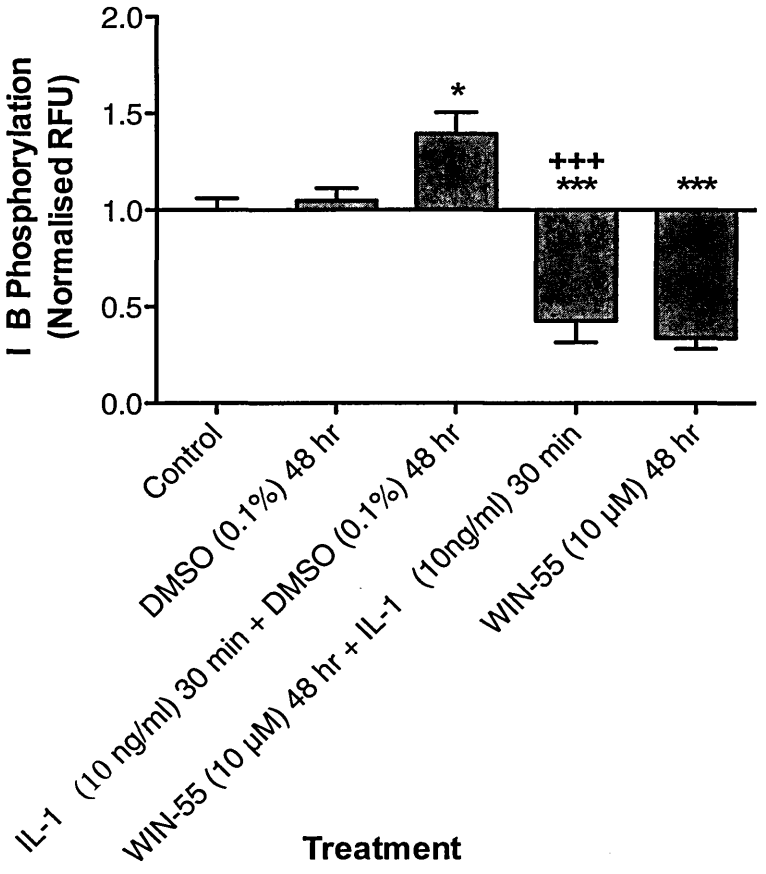

Figure 4.5 The effects of WIN-55 on IL-1β induced IκB phosphorylation. IL-1β stimulation for 30 minutes induced phosphorylation of IκB. WIN-55 pre-treatment for 48 hours in combination with IL-1β for the last 30 minutes significantly reduced phosphorylation of IκB compared to 30 minutes IL-1β stimulation alone. WIN-55 treatment for 48 hours alone significantly reduced IκB phosphorylation below basal levels. Data represents mean fold change of phosphorylation normalised to internal total unphosphorylated protein and untreated control ± SEM. *$p<0.05$, ***$p<0.001$ compared to DMSO control and +++$p<0.001$ compared to IL-1β alone. n=9 obtained from 3 macroscopic grade 3 patients.

4.4.3.3 c-Jun

IL-1β stimulation for 30 minutes significantly increased c-Jun phosphorylation compared to DMSO control ($p<0.001$) (Figure 4.6). Following WIN-55 pre-treatment in combination with IL-1β for the last 30 minutes c-Jun phosphorylation was significantly reduced to basal levels compared to IL-1β treatment alone ($p<0.001$) (Figure 4.6). Phosphorylation of c-Jun remained at basal levels following WIN-55 treatment alone for 48 hours (Figure 4.6).

4.4.3.4 p38

IL-1β stimulation for 30 minutes significantly increased p38 phosphorylation compared to DMSO control ($p<0.001$) (Figure 4.7). WIN-55 pre-treatment for 48 hours in combination with IL-1β for the last 30 minutes did not completely counteract the effects of IL-1β alone and p38 phosphorylation remained significantly higher than DMSO control ($p<0.05$) (Figure 4.7). Phosphorylation of p38 remained at basal levels following WIN-55 treatment alone for 48 hours (Figure 4.7).

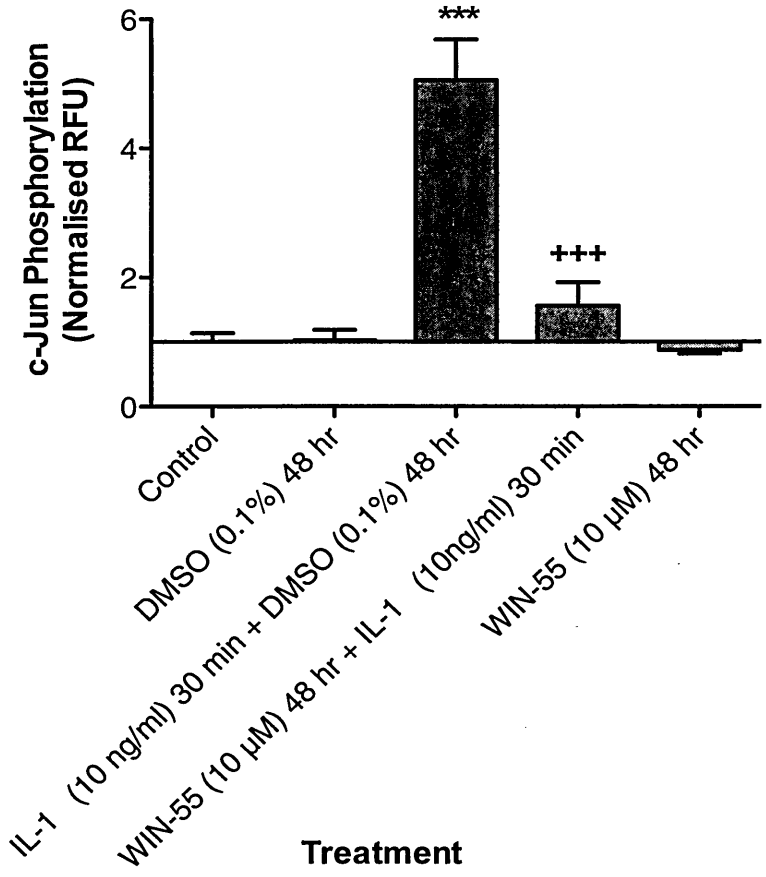

Figure 4.6 The effects of WIN-55 on IL-1β induced c-Jun phosphorylation. IL-1β stimulation for 30 minutes induced phosphorylation of c-Jun. WIN-55 pre-treatment for 48 hours in combination with IL-1β stimulation for the last 30 minutes significantly reduced phosphorylation of c-Jun to basal levels compared to 30 minute IL-1β stimulation alone. Following WIN-55 treatment alone for 48 hours c-Jun phosphorylation remained at basal levels. Data represents mean fold change of phosphorylation normalised to internal total unphosphorylated protein and untreated control ± SEM. ***$p<0.001$ compared to DMSO control and +++$p<0.001$ compared to IL-1β alone. n=9 obtained from 3 macroscopic grade 3 patients.

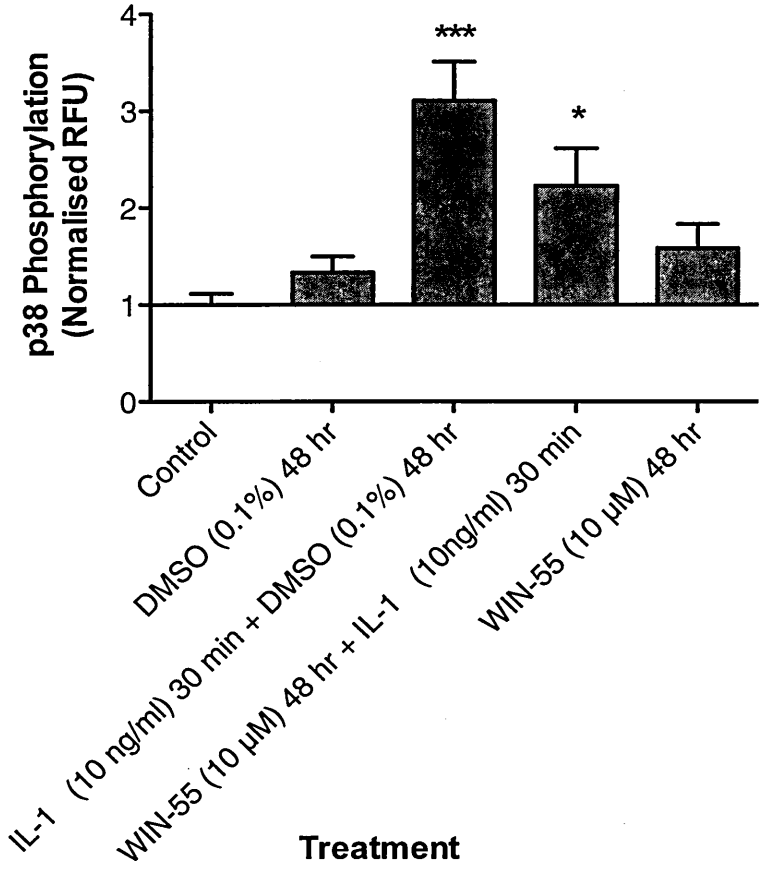

Figure 4.7 The effects of WIN-55 on IL-1β induced p38 phosphorylation. IL-1β stimulation alone for 30 minutes and in combination with WIN-55 for 48 hours induced the phosphorylation of p38. Following WIN-55 treatment alone for 48 hours p38 phosphorylation remained at basal levels. Data represents mean fold change of phosphorylation normalised to internal total unphosphorylated protein and untreated control ± SEM.*$p<0.05$ ***$p<0.001$ compared to DMSO control. n=9 obtained from 3 macroscopic grade 3 patients.

4.4.4 The Effects of WIN-55 and IL-1β on the Phosphorylation of Protein Kinases

The overall effects of WIN-55 and IL-1β on the phosphorylation of 46 different protein kinases are shown in Table 4.3. WIN-55 and IL-1β treatment both alone or in combination induced significant effects on HSP27, c-Jun, ERK1/2, Hck, Fyn, Stat2 and Stat5a protein kinases (Figure 4.8-4.10).

IL-1β stimulation for 30 minutes significantly induced the phosphorylation of HSP27 and c-Jun ($p<0.05$) and decreased the phosphorylation of ERK1/ERK2 ($p<0.01$) compared to DMSO control (Figure 4.8). WIN-55 pre-treatment for 48 hours in combination with IL-1β for the last 30 minutes significantly induced the phosphorylation of HSP27 and c-Jun phosphorylation ($p<0.01$) compared to DMSO control (Figure 4.8). WIN-55 pre-treatment for 48 hours in combination with IL-1β for the last 30 minutes significantly decreased the phosphorylation of ERK1/ERK2 ($p<0.05$) compared to DMSO control. HSP27 and c-Jun phosphorylation remained at basal levels following WIN-55 treatment alone for 48 hours and ERK1/ERK2 phosphorylation was significantly reduced below basal levels compared to DMSO control ($p<0.001$) (Figure 4.8).

IL-1β stimulation for 30 minutes reduced the phosphorylation of Hck, though not significantly and significantly reduced that of Fyn ($p<0.01$) compared to DMSO control (Figure 4.9). Phosphorylation of Fyn and Hck remained at basal levels following WIN-55 pre-treatment for 48 hours in combination with IL-1β treatment for the last 30 minutes; however Fyn phosphorylation was significantly increased compared to IL-1β stimulation alone ($p<0.05$) (Figure 4.9). WIN-55 treatment alone for 48 hours induced the phosphorylation of Hck ($p<0.05$) but not Fyn compared to DMSO control (Figure 4.9).

Following IL-1β stimulation for 30 minutes STAT2 and STAT5a phosphorylation remained at basal levels. WIN-55 pre-treatment in combination with IL-1β for the last 30 minutes significantly reduced STAT2 phosphorylation ($p<0.05$) compared to DMSO control and STAT5a phosphorylation remained at basal levels (Figure 4.10). WIN-55 treatment alone for 48 hours induced a significant increase in STAT5a phosphorylation ($p<0.05$) compared to DMSO control and STAT2 remained at basal levels (Figure 4.10).

Protein Kinase	Phosphorylation site	IL-1β (10 ng/ml)+DMSO (0.1%)	WIN-55 (10 μM)+IL-1β (10 ng/ml)	WIN-55 (10 μM)
p38α	T180/Y182	–	–	–
ERK1/ERK2	T202/Y204, T185/Y187	↓**	↓*	↓***
JNK pan	T183/Y185, T221/Y223	–	–	–
GSK-3 α/β	S21/S9	–	–	–
p53	S392	–	–	–
MEK1/2	S218/S222, S222/S226	–	–	–
MSK1/2	S376/S360	–	–	–
AMPKα1	T174	↓	–	–
Akt	S473	–	–	–
Akt	T308	–	–	–
p53	S46	–	–	–
TOR	S2448	–	–	–
CREB	S133	↓	↑	↑
HSP27	S78/S82	↑*	↑*	–
AMPKα2	T172	–	–	–
B-Catenin	-----	–	–	–

p70 S6 Kinase	T389	–	–	–
p53	S15	–	–	–
p27	T198	–	–	–
Paxillin	Y118	–	–	–
Src	Y419	–	–	–
Lyn	Y397	–	–	–
Lck	Y394	–	–	–
STAT2	Y689	–	↓*	–
STAT5a	Y694	–	–	↑*
p70 S6 Kinase	T421/S424	–	–	–
RSK1/2/3	S380/S386/S377	–	–	–
p27	T157	–	–	–
PLCγ-1	Y783	–	–	–
Fyn	Y420	↓**	–	–
Yes	Y426	–	–	–
Fgr	Y412	–	–	↑
STAT3	Y705	–	–	–
STAT5b	Y699	–	–	–
p70 S6 Kinase	T229	–	–	–

RSK1/2	S221/S227	–	–	–
c-Jun	S63	↑*	↑*	–
Pyk2	Y402	–	–	–
Hck	Y411	↓	–	↑*
Chk-2	T68	–	–	↑
FAK	Y398	–	–	–
STAT6	Y641	–	–	–
STAT5a/b	Y694/Y699	–	–	–
STAT1	Y701	–	↑	–
STAT4	Y693	–	↑	–
eNOS	S1177	–	–	–

Table 4.3 The effects of WIN-55 and IL-1β on the phosphorylation of 46 protein kinases. – Indicates no change in phosphorylation levels compared to DMSO control. ↑ Indicates increase in phosphorylation levels compared to DMSO control. ↓ Indicates decrease in phosphorylation levels compared to DMSO control. *p<0.05, **p<0.01 and *** p<0.001 compared to DMSO control.

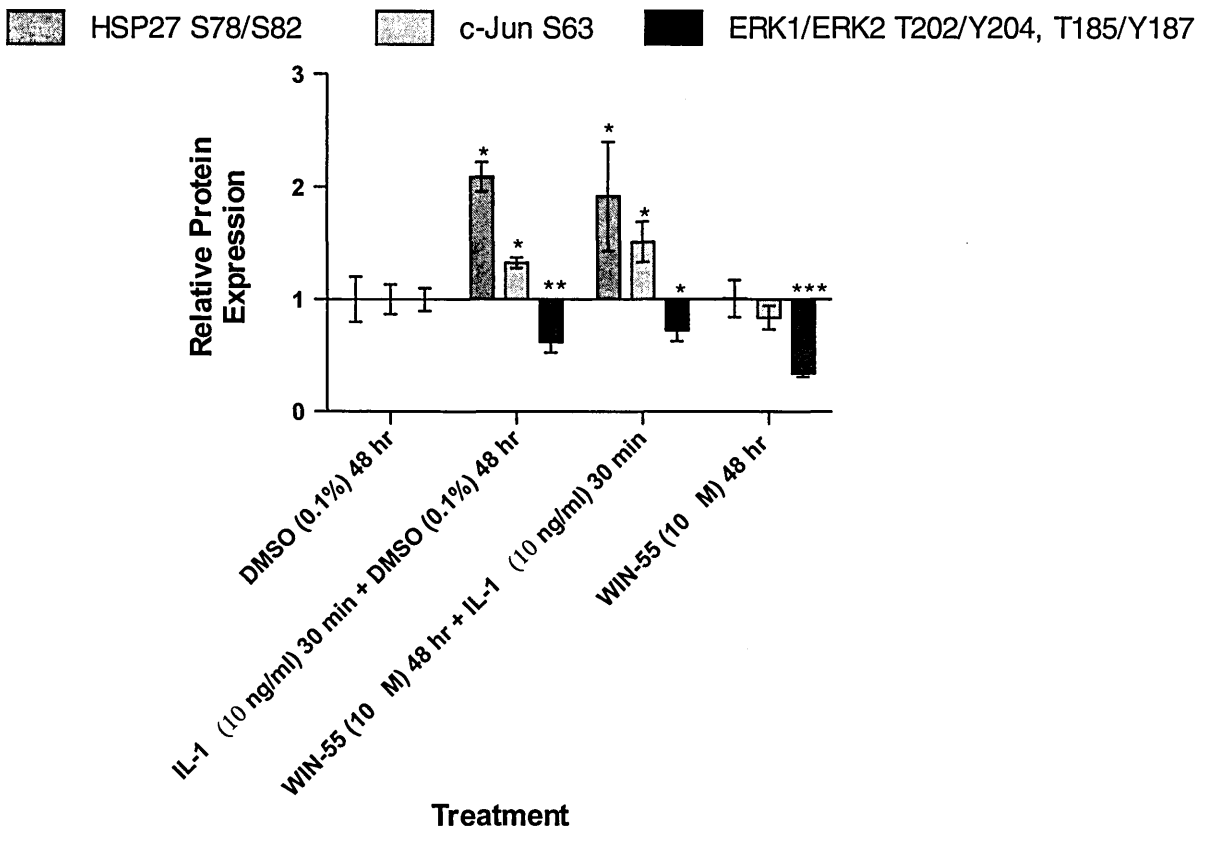

Figure 4.8 The effect of WIN-55 and IL-1β on HSP27, c-Jun and ERK1/ERK2 phosphorylation. IL-1β stimulation for 30 minutes induced the phosphorylation of HSP27 and c-Jun and decreased the phosphorylation of ERK1/ERK2. WIN-55 pre-treatment for 48 hours in combination with IL-1β for the last 30 minutes induced the phosphorylation of HSP27 and c-Jun and decreased the phosphorylation of ERK1/ERK2. HSP27 and c-Jun phosphorylation remained at basal levels and ERK/12 phosphorylation was reduced following WIN-55 treatment alone for 48 hours. Data represents mean fold change of phosphorylation normalised to internal positive control protein and untreated control ± SEM *$p<0.05$ **$p<0.01$ and $p<0.001$ compared to DMSO control. n=4 obtained from one patient sample (HC22(4)).

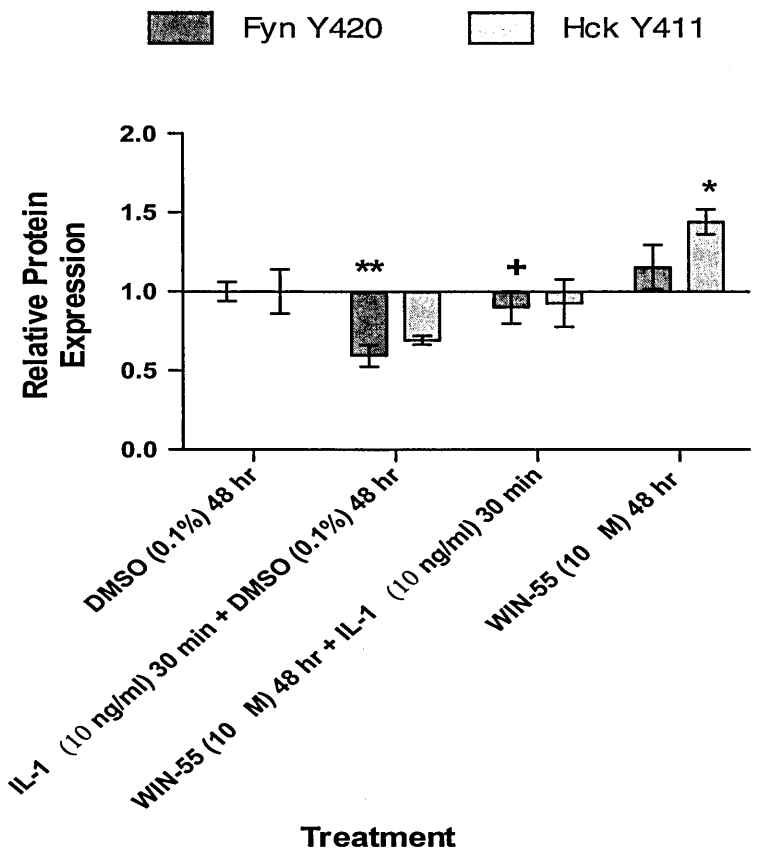

Figure 4.9 The effects of WIN-55 and IL-1β on Fyn and Hck. IL-1β stimulation for 30 minutes reduced the phosphorylation of Fyn and Hck remained at basal levels. Phosphorylation of Fyn and Hck remained at basal levels following WIN-55 pre-treatment for 48 hours in combination with IL-1β stimulation for the last 30 minutes. WIN-55 treatment alone for 48 hours induced the phosphorylation of Hck but not Fyn. Data represents mean fold change of phosphorylation normalised to internal positive control protein and untreated control ± SEM *p<0.05, **p<0.01 compared to DMSO control and +p<0.05 compared to IL-1β treatment alone. n=4 obtained from one patient sample (HC22(4)).

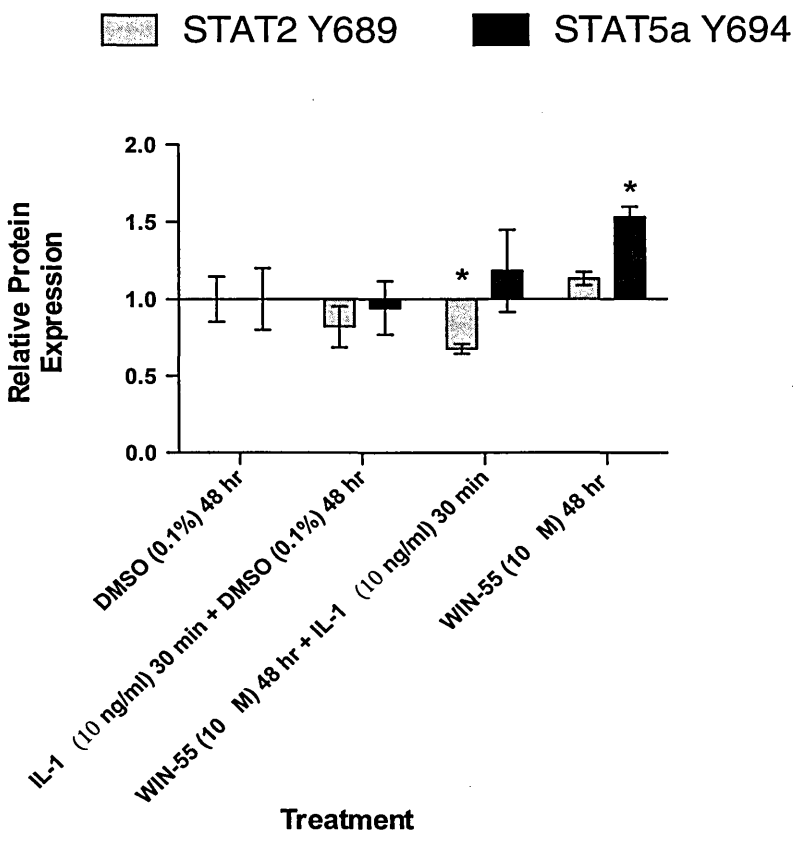

Figure 4.10 The effects of WIN-55 and IL-1β on STAT2 and STAT5a phosphorylation. Following IL-1β stimulation for 30 minutes STAT2 and STAT5a phosphorylation remained at basal levels. WIN-55 pre-treatment for 48 hours in combination with IL-1β for the last 30 minutes reduced STAT2 phosphorylation and STAT5a phosphorylation remained at basal levels. WIN-55 treatment alone for 48 hours induced STAT5a phosphorylation and STAT2 phosphorylation remained at basal levels. Data represents mean fold change of phosphorylation normalised to internal positive control protein and untreated control ± SEM *$p<0.05$ compared to DMSO control. n=4 obtained from one patient sample (HC24(4)).

4.5 Discussion

This work aimed to identify a mechanism via which WIN-55 may act to decrease IL-1β induced expression of MMP-3 and -13 and their inhibitors TIMP-1 and 2. The intracellular signalling cascades initiated by IL-1β upon chondrocyte stimulation ultimately causes the translocation of transcription factors to the nucleus where they induce the expression of target genes involved in the pathogenesis of OA (Vincenti and Brinckerhoff 2002).

4.5.1 NFκB and IκB

Transcription factor NF-κB is activated by IL-1β and induces the expression of MMPs in chondrocytes (Mengshol *et al,* 2000). Liacini *et* al (2002) showed that inhibition of IL-1β induced NF-κB pathway reduced the expression of MMP-3 and MMP-13 in human and bovine chondrocytes (Liacini *et al,* 2002). In the present study, IL-1β increased nuclear immunolocalisation of phosphorylated NFκB following 30 minute stimulation and a 1 hour pre-treatment with WIN-55 did not counteract this effect, but produced phosphorylation of NFκB observed in the cytoplasm of the cell in addition to the nucleus. WIN-55 treatment alone also increased phosphorylation of NFκB however with predominant cytoplasmic immunolocalisation. In previous studies, WIN-55 treatment for 48 hours was shown to inhibit IL-1α induced NF-κB nuclear translocation in bovine chondrocytes (Mbvundula *et al,* 2006). Moreover activation of cannabinoid receptor PPARα using the selective agonist Wy14643 was shown to reduce IL-1β induced NFκB p65 phosphorylation as shown by reduced nuclear immunolocalisation in OA chondrocytes, this response was observed following a 1 hour co-treatment (Clockaerts *et al,* 2011). Furthermore selective inhibition of NFκB in human OA chondrocytes was shown to reduce IL-1β induced expression of MMP-1 and -13 and repression of collagen II (Fan *et al,* 2006).

Other studies have investigated the effect of WIN-55 on NFκB phosphorylation in human astrocytes (Curran *et al,* 2005). Using a reporter gene assay, WIN-55 has been shown to inhibit IL-1β induced activation of NFκB however the exact mechanism by which WIN-55 produced these effects could not be identified (Curran *et al,* 2005). WIN-55 was shown not to inhibit IL-1β induced NFκB translocation to the nucleus and WIN-55 also had no effect on NFκB DNA binding capacity (Curran *et al,* 2005). In addition WIN-55 did not inhibit IL-1β induced degradation of IκBα, concluding that IKKs which directly phosphorylate

IκB, are not direct targets of WIN-55 and that inhibition of NF-κB transactivation by WIN-55 occurs by a novel mechanism (Curran *et al,* 2005). In contrast here it was shown that pre-treatment of chondrocytes for 48 hours with WIN-55 reduced IL-1β induced phosphorylation of IκB, ultimately retaining NFκB in the cytoplasm thus preventing the translocation to the nucleus. Collectively, findings presented here suggest that a 48 hour WIN-55 pre-treatment is required for inhibition of IL-1β induced IκB phosphorylation in human OA chondrocytes. However the mechanism by which WIN-55 mediates these effects remains to be determined.

4.5.2 MAPK signalling pathways

Although inhibition of NF-κB may in part contribute to a decrease in MMP gene expression, other inflammatory pathways induced by IL-1β including MAPKs may be involved and inhibitors of these pathways have been shown to block MMP activity in articular cartilage (Sondergaard *et al,* 2010). During OA IL-1β is up regulated and initiates a cascade of intracellular signalling pathways, which result in the induction of MMPs genes. Liacini *et al* (2002b) showed that inhibition of IL-1β stimulated JNK, p38, ERK1/2 and AP-1 phosphorylation using specific inhibitors resulted in a decrease in MMP-3 and -13 expression in human OA chondrocytes. In a later study Fan *et al* (2006) showed that inhibition of ERK1/2 using specific inhibitors could reduce IL-1β induced expression of MMP-1 and -13 and repression of collagen II. Similarly, inhibition of p38 also resulted in a down-regulation of MMP-1 and -13 but had no effect on collagen II expression (Fan *et al,* 2006). During arthritis there is an increase JNK in response to inflammatory cytokines, which directly phosphorylates c-Jun leading to the induction MMP genes (Vincenti and Brinckerhoff 2002). Interestingly inhibition of JNK signalling had no effect on MMP-1, -3 or collagen II expression in human articular chondrocytes (Fan *et al,* 2006). Since WIN-55 decreased the expression of gene for both MMPs and MMP inhibitors (Chapter 2) it was important to elucidate a possible mechanism via which this may occur.

4.5.2.1 ERK1/ERK2

This study has shown that a 48-hour WIN-55 pre-treatment of human OA chondrocytes was able to counteract IL-1β induced phosphorylation of ERK1/ERK2 using cell based ELISAs. In agreement with this work, WIN-55 was shown to inhibit IL-2 expression via inhibition of ERK1/ERK2 phosphorylation in

mouse splenocytes; furthermore, WIN-55 was shown to reduce inflammatory responses in mouse macrophages by inhibition of ERK1/ERK2 activity; however in contrast to the present study inhibition occurred following 10 minutes treatment (Hao *et al,* 2010). Activation of classical cannabinoid receptors CB1 and CB2 induces phosphorylation of ERK1/ERK2 (Howlett 2005), however in the present study WIN-55 has no effect on basal levels of ERK1/ERK2 at any of the time points investigated (section 4.4.2), suggesting that signalling pathways induced by cannabinoids through GPCRs may be more specific or produce distinctive effects in different types of cells (Luttrell and Luttrell 2003). However, this study has demonstrated that cannabinoid WIN-55 can disrupt IL-1β induced ERK1/ERK 2 signalling pathways and in human OA chondrocytes, thus providing a mechanism for the inhibition of MMP expression (Chapter 2).

4.5.2.2 p38

This study has shown that WIN-55 did not counteract the effects of IL-1β induced p38 phosphorylation. In rat and mouse hippocampus WIN-55 has been shown to induce p38 phosphorylation in a CB1 dependent manner as incubation with CB1 antagonist SR141716A reversed the effects of WIN-55 and stimulatory responses were absent in CB1-/- mice (Derkinderen *et al,* 2001). WIN-55 was also shown to inhibit IL-1β induced p38 phosphorylation in a CB2 dependent manner in human astrocyte cultures (Sheng *et al,* 2009). However, as shown in the present study, in human chondrocytes p38 phosphorylation may not be a direct target of WIN-55. Since each MAPK is regulated differently, depending on substrate specificity and each MAPK can be activated by a number of different MKKs or MKKKs, these findings suggest that WIN-55 may selectively regulate MAPKs (Thalhamer *et al,* 2008).

4.5.2.3 c-Jun

MMP-3, -13 and their inhibitors TIMP-1 and -2 all have promoter regions containing AP-1 transcription factor binding sites, which play a pivotal role in their regulation (Mengshol *et al,* 2000; Vincenti 2001; Borden and Heller 1997; Mengshol *et al,* 2001; Vincenti and Brinckerhoff 2002; Goldring *et al,* 2011). AP-1 complex is composed of fos and Jun families of DNA binding proteins (Karin *et al,* 1997). Fos and Jun proteins form homodimers and heterodimers comprising the AP-1 complex, which binds directly to promoter and enhancer regions on target genes (Karin *et al,* 1997).

Selective JNK inhibitor SP600125 reduced expression of MMP-1 and suppressed c-Jun phosphorylation and AP-1 binding in IL-1β stimulated human fibroblast like synoviocytes (Han *et al,* 2001). In the same study JNK knockout mice showed a decrease in bone and cartilage degradation (Han *et al,* 2001). Together these findings suggest that targeting JNK and subsequently the phosphorylation of c-Jun, may be a therapeutic target in OA. In the present study it was demonstrated that WIN-55 inhibited IL-1β induced phosphorylation of c-Jun in OA chondrocytes using cell based ELISAs. Thus, the WIN-55 induced decrease of MMP-3, -13, TIMP-1 and -2 mRNA expression shown in chapter 2 may be via a decrease in the AP-1 complex, since the abundance of phosphorylated c-Jun directly affects the activity of this transcription factor (Karin *et al,* 1997). Conversely, in HEK293 cells, WIN-55 induced the expression of IFNβ via up regulation of JNK and AP-1; these effects were shown to be dependent on activation of PPARα (Downer *et al,* 2012). In another study using mouse splenocytes, phytocannabinoid CBN inhibited AP-1 binding to the IL-2 promoter region by decreasing the nuclear expression of c-fos and c-Jun (Faubert and Kaminski 2000). Although, AP-1 in part is involved in the transactivation of MMPs and TIMPs, interaction of AP-1 with other transcription factors including epithelial-specific ETS is required (Vincenti 2001). IL-1β induced MMP-13 expression in chondrocytes requires the convergence of AP-1 with ETS/PEA-3 and RUNX (Mengshol *et al,* 2001; Goldring *et al,* 2011). Furthermore, the induction of MMP-13 is thought to be dependent on p38, JNK and NFκB but not ERK1/2 signalling (Mengshol *et al,* 2000; Han *et al,* 2001). Conversely, inhibition of JNK in human articular chondrocytes had no effect on IL-1β induced MMP-13 expression (Fan *et al,* 2006). Interestingly, studies have shown that WIN-55 has no effect on JNK phosphorylation in rat and mouse hippocampus (Derkinderen *et al,* 2001). In order to fully elucidate the effects of WIN-55 on transcription factor activation and binding to target genes involved in OA requires further investigation.

It is important to note, the present study has only determined the phosphorylated amount of ERK1/ERK, c-Jun, p38 and IκB it is therefore possible that IL-1β or WIN-55 may also affect the total amount of these kinases present within the cell.

4.5.3 Protein Kinases

Preliminary studies showed significant changes in phosphorylation levels following IL-1β and WIN-55 stimulation included, ERK1/ERK2, c-Jun, HSP27, Fyn, Hck, STAT2 and STAT5a.

In the present study, proteome array analysis of c-Jun phosphorylation at S63 demonstrated that IL-1β stimulation for 30 minutes induced phosphorylation. These findings are in agreement with cell based ELISA where an increase in c-Jun phosphorylation at the same site following IL-1β stimulation was demonstrated (section 4.4.3.3). However, in contrast to the cell based ELISA methodology (section 4.4.3.1), ERK1/ERK2 phosphorylation at T202/Y204 was decreased following IL-1β stimulation as demonstrated using the proteome array. Furthermore, WIN-55 pre-treatment for 48 hours in combination with IL-1β for 30 minutes induced c-Jun and decreased the phosphorylation of ERK1/ERK2. c-Jun phosphorylation remained at basal levels and ERK/12 phosphorylation was reduced following WIN-55 treatment alone for 48 hours (section 4.4.3.1 and 4.4.3.3). Contrasting findings shown in the present study may be a result of different methods of analysis, in addition the proteome array data was obtained from one patient sample, therefore analysis is required on further patient samples to verify these findings.

Here, HSP27 phosphorylation was induced by IL-1β alone and in combination with WIN-55, with phosphorylation remaining at basal levels when treated with WIN-55 alone. HSP27 is induced by IL-1β and is thought to play a role in IL-1β induced expression of pro-inflammatory mediators involved in OA including IL-6 (Freshney *et al,* 1994; Lambrecht *et al,* 2010). However in the present study IL-1β induced HSP27 phosphorylation was not counteracted by WIN-55 treatment.

Fyn and Hck belong to the Src family of kinases (Bursell *et al,* 2007). IL-1β reduced the phosphorylation of Fyn and slightly reduced the phosphorylation of Hck. The effects of IL-1β were counteracted upon WIN-55 treatment. Fyn phosphorylation remained at basal levels upon WIN-55 treatment alone whilst Hck phosphorylation was induced. To date little is known about the effects of Src kinases in OA, although one study suggested that inhibition of Src might be of therapeutic value in the treatment of skeletal diseases and inhibition of Src in

bovine cartilage explants reduced IL-1β induced MMP production (Sondergaard *et al,* 2010; Bursell *et al,* 2007).

Janus kinases and signal transducer and activator of transcription (JAK/STAT) are a family of transcription factors involved in cytokine induced signalling pathways (Imada and Leonard 2000). In OA, upon chondrocyte stimulation with IL-1β, STAT1 and STAT3 are phosphorylated and translocate to the nucleus where they induce IL-6 expression (Kapoor *et al,* 2011). Although other members of the STAT family have been shown to be involved in the pathogenesis of OA (Kapoor *et al,* 2011), the roles of STAT2 and STAT5a as shown in this study have not been identified. In the present study IL-1β had no effect on STAT2 or STAT5a phosphorylation, WIN-55 in combination with IL-1β reduced STAT2 phosphorylation and WIN-55 alone induced STAT5a phosphorylation, the significance of this remains to be determined.

In order to determine fully the effects of IL-1β and WIN-55 on the protein kinases investigated here using a proteome array would require additional analysis to be performed on further patient samples.

4.5.4 Summary

This study has demonstrated that WIN-55 may prevent IL-1β mediated cartilage breakdown by inhibiting the intracellular phosphorylation of key kinases involved in the induction of MMPs and other catabolic events triggered in OA. Cannabinoid agonists have been shown to signal through MAPK activation upon binding to their respective cannabinoid receptors (Howlett 2005; Demuth and Molleman 2006). In contrast in the present study, WIN-55 did not affect the basal levels of ERK1/2, c-Jun or p38 phosphorylation following 48 hour treatment. MAPK phosphorylation is often used to determine the presence of active G-protein coupled receptors following stimulation with various ligands (Luttrell and Luttrell 2003). Varying results on the signalling pathways cannabinoids induce when obtained from different tissue and cell types suggest that signalling through GPCRs may be more specific or indeed produce distinctive effects in different types of cells (Luttrell and Luttrell 2003).

In the present study a pre-treatment of WIN-55 for 48 hours was required to prevent IL-1β induced c-Jun, p38, ERK1/ERK2 and IκB phosphorylation. Collectively these findings suggest that WIN-55 indirectly inhibits the

phosphorylation of kinases investigated here via the modulation of a yet unidentified pathway possibly by the increase in synthesis of a specific protein over 48 hours, however in order to fully elucidate the mechanism of WIN-55 requires further investigation

5 Cannabinoid Receptor Expression in OA Cartilage

5.1 Introduction

Cannabinoids have been investigated in animal models of arthritis where they have been shown to reduce joint damage and inflammation, suggesting that activation of cannabinoid receptors may be of therapeutic value in the treatment of arthritis (Malfait *et al,* 2000; Sumariwalla *et al,* 2004; Zurier *et al,* 1998; Dunn *et al,* 2012).

During the pathogenesis of arthritis a number of cell types are involved, including synoviocytes and chondrocytes but also bone cells and inflammatory cells (Loeser *et al,* 2012). Cannabinoid receptors have been shown to be expressed within these cell types suggesting these cells are likely to play a role in cannabinoid mediated effects in the arthritic joint (Table 1.3).

Cannabinoids produce their effects by binding to and activating a number of cannabinoid receptors (Pertwee *et al,* 2010). CB1 and CB2 were originally identified as the classical cannabinoid receptors (Matsuda *et al,* 1990; Munro *et al,* 1993). However, it is now apparent that not all physiological effects of cannabinoid receptor ligands are mediated by CB1 and CB2 receptors (Pertwee *et al,* 2010). Phytocannabinoids: CBN and CBD and endogenous cannabinoids OEA and PEA, which are structural analogues of endogenous cannabinoids AEA, display no binding affinity at CB1 or CB2 indicating that these cannabinoids mediate their effects via a non-CB1/CB2 receptor mechanism (Brown 2007). Other receptors including GPR55, GPR18, TRPV1 and PPARs have now been identified as cannabinoid receptors as they have been shown to bind a number of endogenous, synthetic and phytocannabinoids (Pertwee *et al,* 2010).

5.1.1 CB1 and CB2

CB1 and CB2 receptors are expressed by bovine and human articular chondrocytes (Mbvundula *et al,* 2006; Andersson *et al,* 2011) and have been detected in the synovium and fibroblast like synovial cells of OA and RA patients (Richardson *et al,* 2008; Selvi *et al,* 2008). In bovine cartilage, synthetic cannabinoids HU210 and WIN-55 were shown to reduce IL-1α induced proteoglycan and collagen degradation, effects that were thought to be mediated by CB1 and CB2 receptors (Mbvundula *et al,* 2006). Moreover, in OA and RA derived synovial fibroblast like cells WIN-55 was shown to inhibit IL-1β

induced secretion of IL-6 and IL-8 suggesting anti-inflammatory actions are also mediated by CB1 and CB2 receptors (Selvi *et al,* 2008).

5.1.2 GPR55

GPR55 has recently been identified as a cannabinoid receptor, however it has low sequence homology with CB1 and CB2 (McPartland *et al,* 2006; Sharir and Abood 2010). GPR55 is activated by a number of exogenous and endogenous cannabinoid ligands (Ryberg *et al,* 2007; Kapur *et al,* 2009;Johns *et al,* 2007). Moreover, GPR55 is expressed in both normal and OA human chondrocytes (Andersson *et al,* 2011) and abnormal cannabinoid O-1602 was shown to reduce inflammatory pain in a rat model of arthritis, effects that are thought to be mediated by GPR55 (Schuelert and McDougall 2011).

5.1.3 GPR18

Phytocannabinoid THC and endogenous cannabinoids AEA and the AEA metabolite NAGly are ligands of GPR18, suggesting that GPR18 may also act as a cannabinoid receptor (McHugh *et al,* 2012; McHugh *et al,* 2010). GPR18 is primarily expressed in tissues and cells involved in endocrine and immune functions (Gantz *et al,* 1997) and has yet to be identified in articular cartilage chondrocytes or bone cells.

5.1.4 TPRV1

Cannabinoid receptor TRPV1 is expressed by human OA chondrocytes and human OA and RA synovial fibroblasts (Gavenis *et al,* 2009; Engler *et al,* 2007). TRPV1 acts as an endogenous cannabinoid receptor for AEA and has also been shown to bind phytocannabinoid CBD (Bisogno *et al,* 2001; Smart and Jerman 2000). Furthermore, CBD has been shown to have anti-inflammatory and anti-hyperalgesic in a rat model of acute inflammation, effects that were thought to be mediated by TRPV1 (Costa *et al,* 2004; Costa *et al,* 2007).

5.1.5 PPARs

The PPAR nuclear receptor family have been shown to bind a number of different cannabinoid ligands, which are thought to have anti-inflammatory properties mediated primarily by PPARγ activation (O'Sullivan and Kendall 2010). All three subtypes of PPAR are expressed at the mRNA or protein level in rat growth plate chondrocytes and human chondrocytes. (Bordji *et al,* 2000; Shao *et al,* 2005; Fahmi *et al,* 2001; Afif *et al,* 2007; Boyault *et al,* 2001;

Clockaerts *et al,* 2011). PPAR activation by both cannabinoid and non-cannabinoid ligands display chondroprotective activities in both OA and RA (O'Sullivan and Kendall 2010; Fahmi *et al,* 2001; Fahmi *et al,* 2002; Johnson *et al,* 2007; Clockaerts *et al,* 2011; Fahmi *et al,* 2011; Giaginis *et al,* 2009).

Although previous studies have shown that cannabinoids and cannabinoid receptors display chondroprotective properties, the expression and localisation of cannabinoid receptors within OA cartilage and bone is poorly defined. Furthermore, WIN-55 is an agonist at the classical cannabinoid receptors CB1 and CB2, but also has been shown to activate PPARα and γ (Pertwee *et al,* 2010; O'Sullivan and Kendall 2010; Sun *et al,* 2006). WIN-55 may also display activities at TRPV1 (Jeske *et al,* 2006). WIN-55 is thought to display no activities at the GPR55 receptor (Kapur *et al,* 2009) and there is no current knowledge as to its effects at PPARδ or GPR18 receptors. Furthermore, plasma membrane cannabinoid receptors including CB1, CB2, GPR55 and TRPV1, upon ligand binding are known to internalise and undergo cellular redistribution and nuclear receptors PPARα and γ have also been shown to shuttle between the nucleus and cytoplasm following external stimulation (Kapur *et al,* 2009; Hsieh *et al,* 1999; Shenoy and Lefkowitz 2003; Abood 2005; Atwood *et al,* 2012; Sanz-Salvador *et al,* 2012; Umemoto and Fujiki 2012), suggesting that a process of cellular redistribution may regulate or contribute to the effects of cannabinoid receptor mediated chondroprotection.

5.2 Aims and Objectives

Aim: To investigate the expression and modulation of cannabinoid receptors in human OA cartilage.

Objectives:

- To investigate the expression of cannabinoid receptors CB1, CB2, GPR55, GPR18, TRPV1 and PPARα δ and γ within different microscopic grades of OA cartilage immunohistochemically.
- To determine if cannabinoid receptor expression was grade and cartilage zone specific.
- To determine the effects of WIN-55 on CB1, CB2, GPR55, GPR18, TRPV1 and PPARα, δ and γ receptor expression and localisation in chondrocyte monolayer cultures.
- To determine the effects of WIN-55 and IL-1β on PPARα, δ and γ mRNA expression in chondrocytes in monolayer culture.

5.3 Experimental design

Cartilage tissue blocks obtained from each anatomic compartment within the knee (medial and lateral tibio-femoral and patello-femoral compartments) (See Appendix 1) were used to investigate the expression and localisation of cannabinoid receptors CB1, CB2, GPR55, GPR18, TRPV1 and PPARα, δ and γ within different microscopic grades of OA cartilage. Tissue was fixed, processed to paraffin and cut into sections. Sections were stained with haematoxylin and eosin, alcian blue and Masson Trichrome for microscopic grading. OA cartilage was graded microscopically and the total score of each cartilage section was determined. Patient samples from each grade were used for immunohistochemical analysis of cannabinoid receptors within chondrocytes in each of the cartilage zones (superficial, middle, deep and clusters) and osteocytes in the bone. In addition ICC was used to investigate the effects of WIN-55 on cannabinoid receptors CB1, CB2, GPR55, GPR18, TRPV1 and PPARα δ and γ expression and localisation in OA chondrocytes in monolayer culture. Finally, the effects of WIN-55 and IL-1β on PPARα, δ and γ mRNA expression, in OA chondrocytes in monolayer culture was investigated using real-time PCR.

5.4 Methodology

5.4.1 Paraffin-Wax Embedding of Cartilage Tissue

Cartilage blocks were fixed in 10% formalin (Sigma-Aldrich,UK) followed by EDTA decalcification (Leica). Cartilage samples were processed to wax using an automated process with graded solutions of industrial methylated spirits (IMS;Fisher Scientific), used to dehydrate samples followed by hydration in SUB-X solution (Leica). Cartilage samples were immersed in molten paraffin wax twice; full wax processing details are given in Appendix 3. Samples were transferred to fresh molten wax in a JeioTech OV-11 vacuum oven and incubated at 60°C and 60 cmHg for 1 hour to allow for complete penetration of wax through the cartilage samples. Cartilage samples were then transferred to moulds containing molten wax and orientated as desired and the wax allowed to set on a cold plate to form blocks. Tissue blocks were removed from moulds and stored at room temperature prior to sectioning as described in section 5.4.2.

5.4.2 Sectioning and Mounting

Tissue sections were cut using a Leica SM2400 sledge microtome to 4 µm thickness. Tissue sections were placed onto water containing 0.01% v/v gelatin solution (Sigma-Aldrich) at 45°C and mounted onto positively charged slides (Leica). Sections were allowed to dry on a drying rack at 40°C and were then transferred to an oven at 37°C for further drying until use.

5.4.3 Haematoxylin and Eosin

Sections were de-waxed in SUB-X (Leica) for 5 minutes in triplicate, rehydrated in IMS (Fisher Scientific) for 4 minutes in triplicate and immersed in deionised water for 5 minutes. Sections were stained with Mayer's Haematoxylin (Leica) for 1 minute and blued in running tap water for 5 minutes. Sections were then counterstained in eosin (Leica) for 1 minute, dehydrated in IMS for 4 minutes in triplicate and cleared in SUB-X (Leica) for 5 minutes in triplicate. Sections were mounted in Pertex (Leica).

5.4.4 Alcian Blue Staining

Sections were de-waxed and rehydrated as outlined in section 5.3.3. Tissue sections were stained in 1% w/v acid alcian blue in 3% v/v acetic acid (pH 2.4) (Sigma-Aldrich) for 15 minutes and counter stained in 1% w/v aqueous neutral red in deionised water (Sigma-Aldrich) for 1 minute. Sections were dehydrated

in IMS for 4 minutes in triplicate and cleared in SUB-X (Leica) for 5 minutes in triplicate. Sections were mounted in Pertex (Leica).

5.4.5 Masson Trichrome

Sections were de-waxed and rehydrated as outlined in section 5.4.3. Sections were stained with Masson Trichrome (Leica) according to the manufacturer's instructions. Sections were dehydrated in IMS for 4 minutes in triplicate and cleared in SUB-X (Leica) for 5 minutes in triplicate. Sections were mounted in Pertex (Leica).

5.4.6 Cartilage Microscopic Grading

Cartilage tissue sections were visualised and graded microscopically using an Olympus BX60 microscope. Each section was given a grade between 0 and 22 based on the presence of histological features that are associated with OA. The method of tissue grading was developed by Dr Christine Le Maitre, using previously published histological grading methods (Mankin *et al,* 1971;Pritzker *et al,* 2006). A score of 0-6 indicated non-degenerate cartilage, 7-12 low-degeneration, 13-17 intermediate degeneration and 18-22 severe degeneration. Two observer's graded all sections independently and the average of the two observers grades was assigned to each cartilage tissue section. Sections were graded on the basis of different histological features including the structure and thickness of the cartilage, the chondrocytes' organisation, the proteoglycan content, the integrity of the tidemark and abnormal features including bone remodelling, as shown in Table 4.1. The histological appearance of cartilage tissue following staining as outlined in sections 5.4.3, 5.4.4 and 5.4.5 obtained from non-degenerate, low-degeneration, intermediate degeneration and severe degeneration can be seen in Figures 5.1 to 5.4.

Item	Classification	Score
Structure (H&E/MT)	Normal	0
	Superficial fibrillation surface irregularities	1
	Pannus and surface irregularities	2
	Clefts to Transitional zone	3
	Clefts to Radial zone	4
	Clefts to Calcified zone	5
	Complete disorganisation	6
Cells (H&E/MT)	Normal	0
	Diffuse hypercellularity (<25%)	1
	Clusters (25-75%)	2
	Hypocellularity (>75%)	3
Proteoglycan content (Alcian Blue)	Normal	0
	Slight reduction (surface zone loss)	1
	Moderate reduction (Upper 1/3 loss)	2
	Severe reduction (Into upper 2/3 deep zone)	3
	No staining present or very limited in bottom 1/3	4
Tidemark Integrity (H&E/MT)	Intact	0
	Crossed by blood vessels	1
Abnormal features (H&E/MT)	None	0
	Denudation: Surface sclerotic bone or reparative tissue including fibrocartilage microfractures with limited repair to bone surface	2
	Deformation: Bone remodelling (more than osteophyte formation) Includes microfracture with fibrocartilaginous and osseous repair extending above previous surface	4
Cartilage thickness (H&E/MT)	Normal smooth articulating surface	0
	Thinning of superficial zone	1
	Thinning into Middle Zone (>25% surface area)	2
	Thinning into Deep Zone (>25% surface area)	3
	Areas where bone exposed (>25% surface area)	4

Table 5.1 Histological Grading of Cartilage Sections. H&E; haematoxylin and eosin; MT; Masson Trichrome

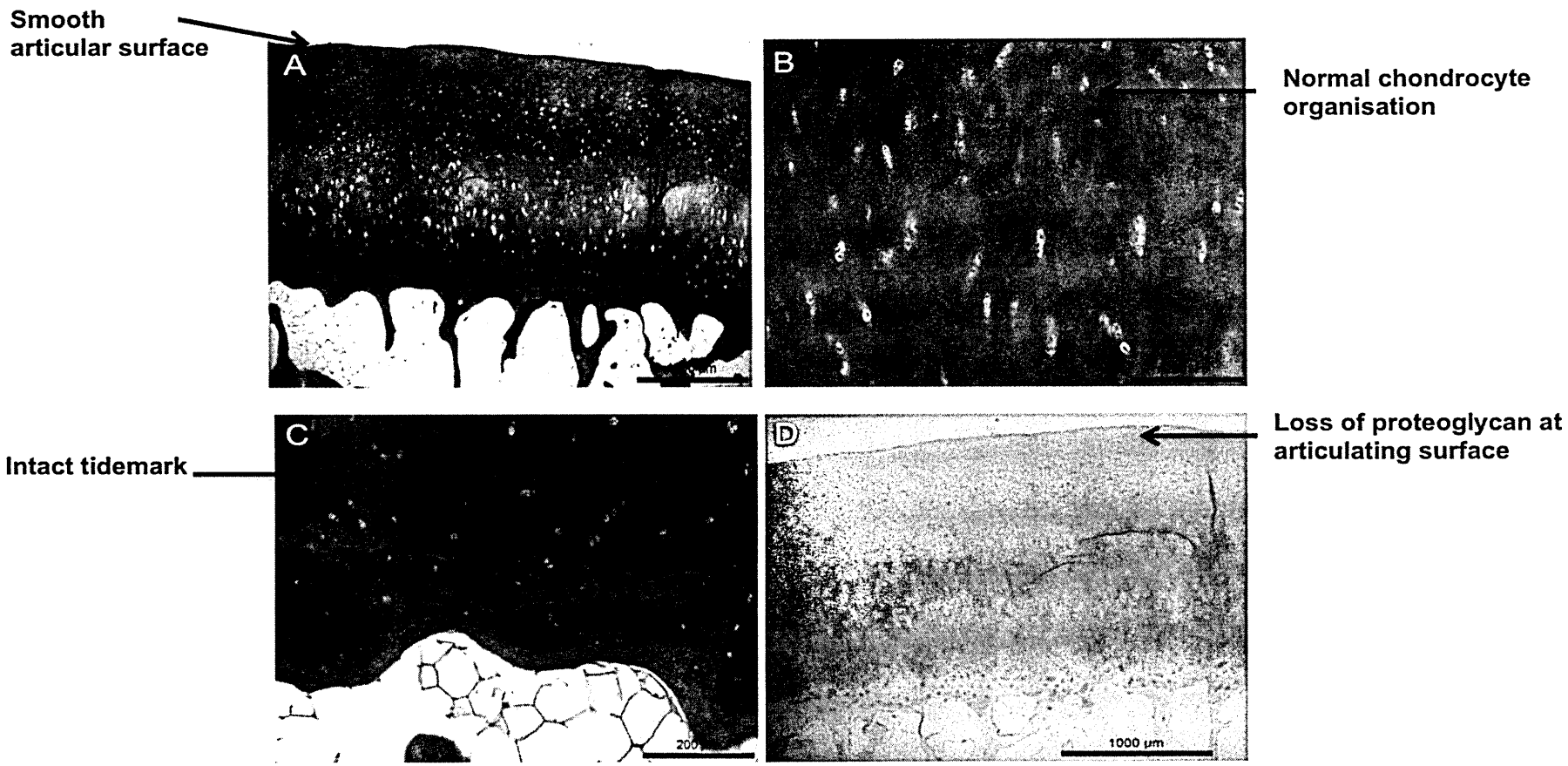

Figure 5.1 Histological features of non-degenerate cartilage samples (HC19(1)). (A-C) Masson Trichrome. **(D)** Alcian Blue. Histological features of non-degenerate cartilage (Score 0-6) include normal cartilage structure with a smooth articulating surface **(A).** The chondrocytes are organised into superficial, middle and deep zones **(A&B).** The tidemark is intact and the bone appears normal **(C).** The proteoglycan content is normal, with small loss of proteoglycan at the articulating surface sometimes present **(D).** Scale Bars (A) 1000 µm, (B) 200 µm, (C) 200 µm, (D) 1000 µm

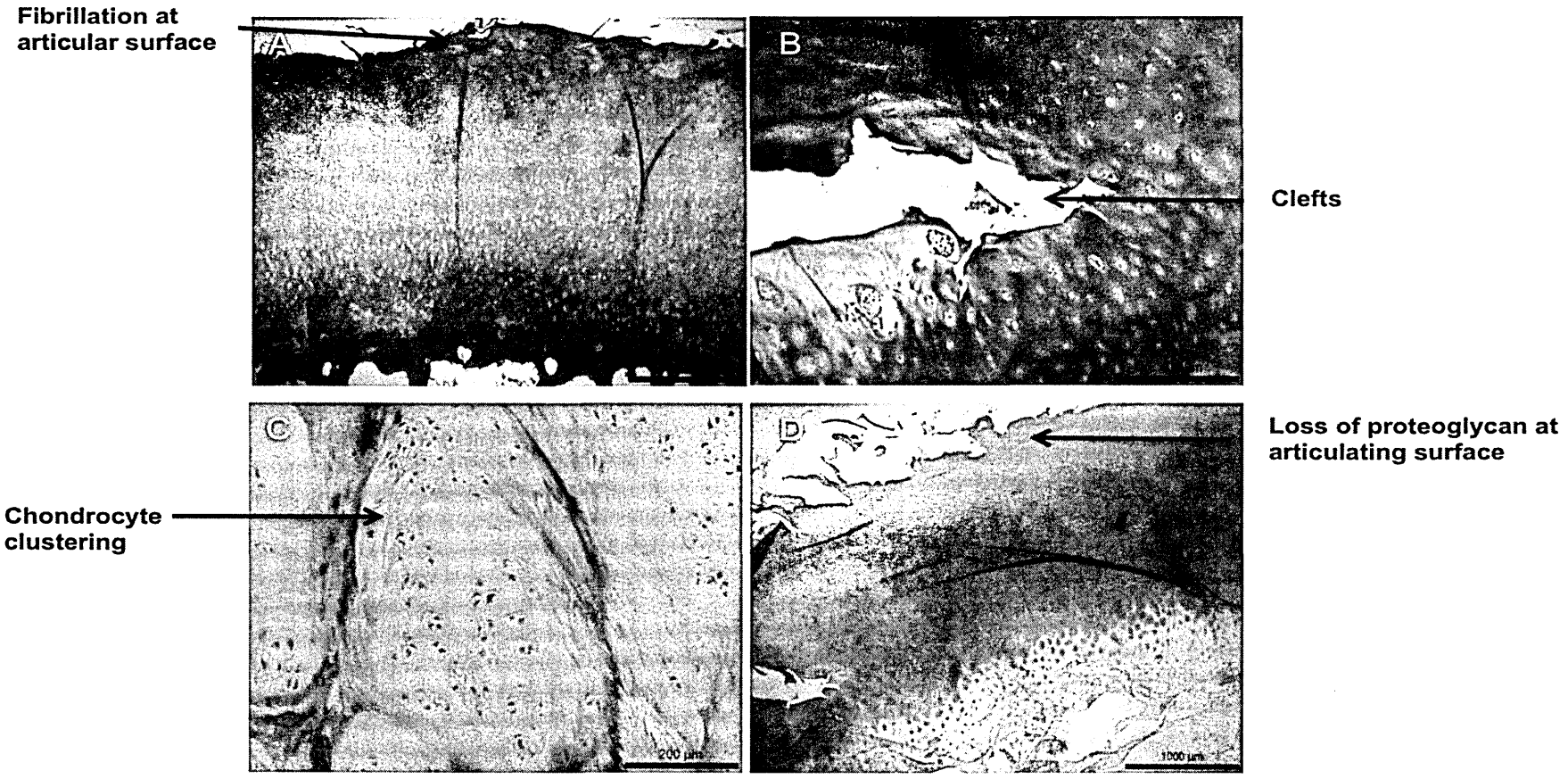

Figure 5.2 The histological features of low grade degeneration (HC9(3)). (A-C) Masson Trichrome. **(D)** Alcian Blue. Histological features of low-grade degeneration include, superficial fibrillation to the articular surface and clefts into the cartilage may be present **(A).** The chondrocytes proliferate and clusters may be present **(C).** Proteoglycan depletion is observed at articular surface **(D).** Scale Bars (A) 1000 µm, (B) 400 µm, (C) 200 µm, (D) 1000 µm

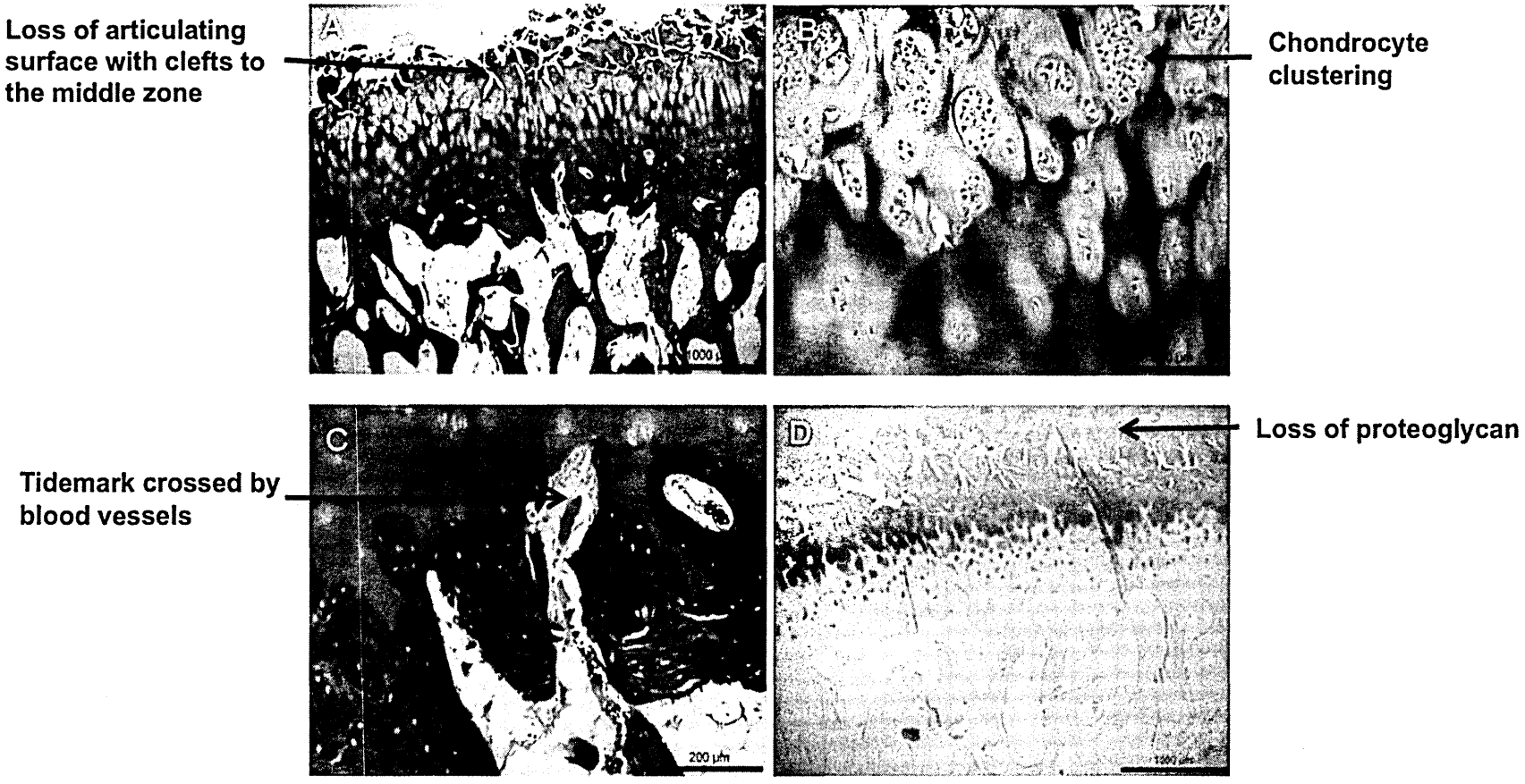

Figure 5.3 The histological features of intermediate degeneration (HC5(4)). (A-C) Masson Trichrome. (D) Alcian Blue. Histological features of intermediate degeneration include loss of articulating surface with clefts to the middle and transitional zone **(A).** This is accompanied by clustering and proliferation of chondrocytes throughout the cartilage **(B).** The tidemark is crossed by blood vessels and there is increased loss of proteoglycan **(C&D).** Scale Bars (A) 1000 µm, (B) 200 µm, (C) 200 µm, (D) 1000 µm

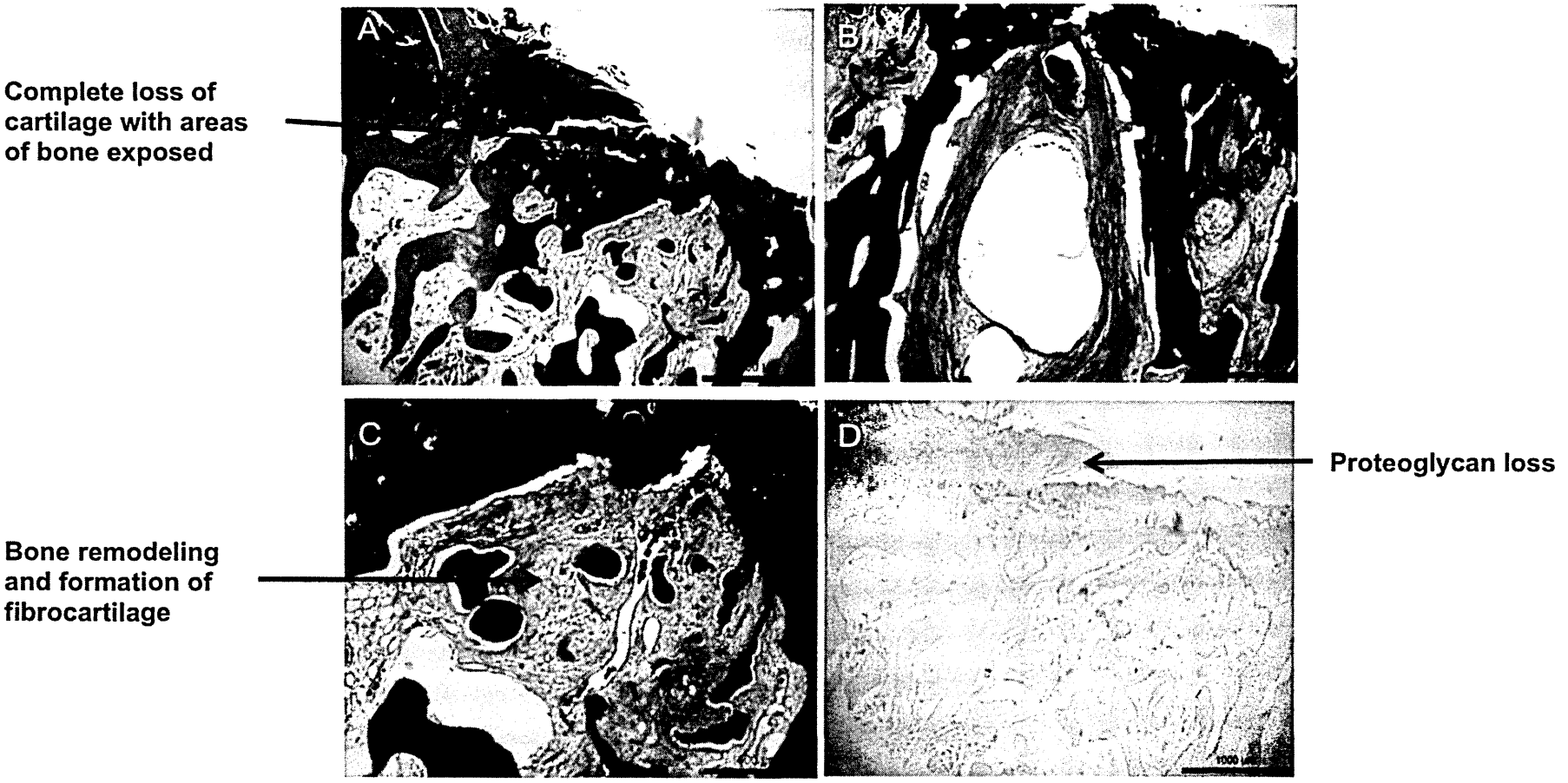

Figure 5.4 The histological features of severe degeneration (HC15(3)). (A-C) Masson Trichrome. **(D)** Alcian Blue. Histological features of severe degeneration include complete loss of cartilage with areas of bone exposed **(A).** Remodelling of bone and the formation of fibrocartilage is present **(B&C).** In addition complete depletion of proteoglycans is observed **(D).** Scale Bars (A) 1000 µm, (B) 1000 µm, (C) 400 µm, (D) 1000 µm

5.4.7 Immunohistochemistry (IHC)

5.4.7.1 The principle of IHC

IHC is a technique, which uses antibodies to detected target antigens in tissue. A primary antibody is incubated with the tissue followed by the addition of a secondary antibody, which is directed against the species of the primary antibody. The secondary antibody is conjugated to biotin, an enzyme or fluorophore. The conjugated label allows for the detection and localisation of the bound primary antibody within the tissue, which can be visualised by the addition of a substrate or by fluorescent emission.

5.4.7.2 IHC using Streptavidin-Biotin-HRP DAB detection

This study used streptavidin-biotin-HRP 3,3'-diaminobenzidine tetrahydrochloride (DAB) detection in IHC for the visualisation and localisation of cannabinoid receptors within OA cartilage and bone. The secondary antibody used against the bound primary antibody was conjugated to biotin to detect the target antigens. The Avidin/Biotin complex (ABC) reagent was added with horseradish peroxidase bound to the biotinylated secondary antibody formed an enzyme complex. The addition of DAB, a substrate of the enzyme complex, produced a brown polymerised precipitate where the secondary antibody was bound. The endogenous peroxidases present in the tissue were blocked to prevent non-specific staining (section 5.4.12).

5.4.7.3 Antigen Retrieval Optimisation

Tissue fixation, processing and embedding masks antigens within the tissue and affects protein antigenicity. Antigen retrieval (AR) increases the sensitivity of IHC, allowing for improved detection of epitopes. AR may be obtained by heat-based methods or enzymatic epitope retrieval.

In this study a number of different antigen retrieval methods were used including enzymatic and heat treatment of tissue to achieve optimal antigen retrieval (Table 5.2). The optimum method determined and used for all subsequent IHC was enzymatic digestion using 0.01% chymotrypsin.

Antigen Retrieval Method		Assessment of Antigen Retrieval
Heat		
Microwave	Microwave irradiation for 10 minutes in 0.05M Tris-HCl buffer pH 9.5	Tissue detached from slide
Steamer	Incubation in steamer in 0.01M citrate buffer pH 6 for 20 minutes	Tissue detached from slide
Enzymatic		
Chymotrypsin	Incubation in 0.01% chymotrypsin in 1xTBS at 37 °C for 20 minutes	Improved AR and no damage to tissue.
Hyaluronidase	Incubation in 10 µg/ml hyaluronidase in 1xTBS at 37 °C for 30 minutes	Poor AR

Table 5.2 Optimisation of Antigen retrieval. Different methods of antigen retrieval were investigated on formalin fixed and paraffin embedded cartilage tissue. Incubation of sections in chymotrypsin produced improved antigenicity so was used for all IHC (section 5.4.9)

5.4.8 Titration of Primary Antibodies

In order to achieve optimal IHC staining without background or non-specific staining, primary antibody titration is required for each application and each set of experimental conditions. In this study each primary antibody was titrated to determine the optimal dilution for the experimental conditions (Table 5.3).

Target Antigen	Clonality	Titration Range	Optimal Concentration (Dilution)
CB1 (Abcam)	Rabbit Polyclonal	1:25-1:500	1:100 (8 µg)
CB2 (Abcam)	Rabbit Polyclonal	1:25-1:1000	1:50 (10 µg)
GPR55 (Acris)	Rabbit Polyclonal	1:50-1:1000	1:100 (10 µg)
GPR18 (Acris)	Rabbit Polyclonal	1:50-1:1000	1:200 (2.5 µg)
TRPV1 (Abcam)	Rabbit Polyclonal	1:250-1:3000	1:1000 (Concentration not determined)
PPARα (Abcam)	Rabbit Polyclonal	1:50 -1:1000	1:250 (4 µg)
PPARδ (Abcam)	Rabbit Polyclonal	1:50-1:1000	1:150 (6.6 µg)
PPARγ (Abcam)	Rabbit Polyclonal	1:25-1:500	1:50 (4 ug)

Table 5.3 Primary Antibodies. The optimal concentration of the primary antibodies was determined by titration

5.4.9 Isotype, Negative and Labelling Controls

In addition to primary antibody titration (section 5.3.10), IgG control tissue sections were run for each primary antibody at the same protein concentration. The absence of staining in the IgG control tissue sections confirmed the specificity of the primary antibody.

For negative control sections, primary antibody was replaced with 1% BSA in 1xTBS. The absence of staining in these tissue sections confirmed that the binding of the secondary antibody was specific to the primary antibody.

For labelling controls, both primary and secondary antibodies were replaced with 1% BSA in 1xTBS. The absence of staining in these tissue sections confirmed that immunopositivity observed was not a result of the presence of endogenous peroxidases present.

5.4.10 IHC Methodology

Cartilage samples were sectioned and mounted as outline in section 5.3.2. Cartilage samples used for IHC are shown in Table 5.4. Sections were de-waxed and rehydrated as outlined in section 5.3.4. Sections were incubated in 3% v/v hydrogen peroxide (Sigma-Aldrich) in IMS (Fisher-Scientific) for 30 minutes to block endogenous peroxidases. Sections were washed in deionised water for 5 minutes followed by two washed in 1xTBS.

5.4.11 Antigen Retrieval

Sections were incubated with 1xTBS which had been pre-warmed to 37°C prior to incubation in 0.01% w/v chymotrypsin (Sigma-Aldrich) with 0.1% w/v $CaCl_2$ solution in 1xTBS for 20 minutes at 37°C. Sections were washed in 1xTBS to inactivate the chymotrypsin.

5.4.12 Binding of Primary and Secondary Antibodies

Non-specific binding sites were blocked as described in section 2.3.12. Blocking solution was removed and primary antibodies were diluted in 1% w/v BSA in 1xTBS (Table 5.3) and 100 µl of primary antibody, isotype control or blocking solution alone, which served as the negative control were applied to the sections and incubated on the sections overnight at 4°C. The optimal concentration for each primary antibody was determined by titration as described in section 5.3.10 (Table 5.3). Sections were washed three times in 1xTBS and 100 µl of biotinylated goat anti rabbit secondary antibody (Abcam) diluted 1/300 in 1% w/v BSA in 1xTBS was applied to each section and incubated at room temperature for 30 minutes.

5.4.13 Detection of Secondary Antibodies

Sections were washed three times in 1xTBS and 2 drops of A.B.C Elite Reagent (Vector Laboratories) was applied to each section and incubated at room temperature for 30 minutes. Sections were washed three times in 1xTBS and 100 µl of DAB (Sigma-Aldrich) solution containing 0.03% v/v hydrogen peroxide (Sigma-Aldrich) was applied to each section and incubated at room temperature for 20 minutes. Sections were washed in deionised water for 2 minutes and then counterstained in Mayer's Haematoxylin (Leica) for 1 minute and blued in running water for 5 minutes.

Microscopic Grade	Cartilage Sample ID	Microscopic Score	Receptor Expression Investigated							
			CB1	CB2	GPR55	GRP18	TRPV1	PPARα	PPARδ	PPARγ
Non-degenerate (0-6)	HC4(1)	4	✓	✓	✓	✓	✓	✓	✓	✓
	HC6(2)	5	✓	✓	✓	✓	✓	✓	✓	✓
	HC9(1)	2.5	✓	✓	✓	✓	✓	✓	✓	✓
	HC10(1)	5	✓	✓	×	✓	✓	✓	✓	✓
	HC11(1)	3.5	×	×	✓	✓	×	×	×	×
	HC11(2)	4	✓	✓	✓	✓	✓	✓	✓	✓
	HC11(4)	5	✓	✓	✓	✓	✓	✓	✓	✓
	HC17(1)	3	✓	✓	✓	✓	✓	✓	✓	✓
	HC18(1)	3	✓	✓	✓	×	✓	✓	✓	✓
	HC19(1)	5	✓	✓	✓	✓	✓	✓	✓	✓
Low degeneration (7-12)	HC3(6)*	8	✓	✓	✓	✓	✓	✓	✓	✓
	HC4(3)	11	✓	✓	✓	✓	✓	✓	✓	✓
	HC5(5)	9	✓	✓	✓	✓	✓	✓	✓	✓
	HC6(4)	8.5	✓	✓	✓	✓	✓	✓	✓	✓
	HC18(4)	9	✓	✓	✓	✓	✓	✓	✓	✓
	HC9(3)*	10	✓	✓	✓	×	✓	✓	✓	✓
	HC13(2)	10	✓	✓	✓	✓	✓	×	✓	✓
	HC16(2)	8.5	×	✓	×	✓	✓	✓	✓	✓
	HC19(2)	9.5	✓	✓	✓	✓	✓	✓	✓	✓

Microscopic Grade	Cartilage Sample ID	Microscopic Score	Receptor Expression Investigated							
			CB1	CB2	GPR55	GRP18	TRPV1	PPARα	PPARδ	PPARγ
Intermediate degeneration (13-17)	HC2(3)*	15	✓	✓	✓	✓	✓	✓	✓	✓
	HC13(4)*	16.5	✓	✓	✓	✓	✓	✓	✓	✓
	HC4(6)*	13.5	✓	✓	✓	✓	✓	✓	✓	✓
	HC5(4)*	16	✓	✓	✓	✓	✓	✓	✓	✓
	HC5(7)*	14.5	✓	✓	✓	✓	✓	✓	✓	✓
	HC6(3)*	14.5	✓	✓	✓	✓	✓	✓	✓	✓
	HC6(5)*	14.5	✓	✓	✓	✓	✓	✓	✓	✓
	HC14(3)*	14.5	✓	✓	✓	×	×	✓	✓	✓
	HC16(6)	13.5	✓	✓	✓	✓	✓	✓	✓	✓
Severe degeneration (18-22)	HC10(6)	19.5	✓	✓	✓	✓	✓	✓	✓	✓
	HC6(6)*	21	✓	✓	✓	✓	✓	✓	✓	✓
	HC7(6)	22	✓	✓	✓	✓	✓	✓	✓	✓
	HC9(7)*	21.5	✓	✓	✓	✓	✓	✓	✓	✓
	HC11(6)	19.5	✓	✓	✓	✓	✓	✓	✓	✓
	HC12(5)	20.5	✓	✓	✓	✓	✓	✓	✓	✓
	HC15(3)	21.5	✓	✓	✓	✓	✓	✓	✓	✓
	HC15(4)*	19.5	✓	✓	✓	✓	✓	✓	✓	✓
	HC17(6)*	17.5	✓	✓	✓	✓	✓	✓	✓	✓
	HC19(3)	17.5	✓	✓	✓	✓	✓	✓	✓	✓

Table 5.4 Tissue samples used for IHC of cannabinoid receptors. Full details of patient samples can be found in Appendix 1. Each tissue section was graded microscopically following histological staining as described in section 5.4.3-5.4.5. *Indicates samples where cellular clusters were present.

5.4.14 Mounting of Sections

Sections were dehydrated, cleared and mounted as described in section 5.4.3.

5.4.15 Microscopy and Image Capture

All cartilage tissue sections were visualised using the Olympus BX60 microscope and images were captured using the QCapture Pro V8.0 software (MediaCybernetics).

5.4.16 Analysis of Immunohistochemistry

Percentage immunopositivity of target antigens was determined by counting 200 cells in each of the cartilage zones as shown in Figure 5.5.

5.4.17 Statistical Analysis

Data was combined for each of the tissue zones and for non-degenerate, low degeneration, intermediate degeneration and severe degeneration. The data obtained was non-parametric, so Kruskal Wallis with Dunn's Multiple Comparisons Test or Mann Whitney statistical testing was used to compare between non-degenerate, low degeneration, intermediate degeneration and severe degeneration sample groups within the different zones of tissue. Data was represented graphically and statistical testing was performed using Prism v5 (GraphPad Software Inc).

Linear regression analysis was used to identify correlation between percentage immunopositivity and microscopic grade of degeneration determined histologically. Data was represented graphically and Linear Regression analysis was performed using Prism v5 (GraphPad Software Inc).

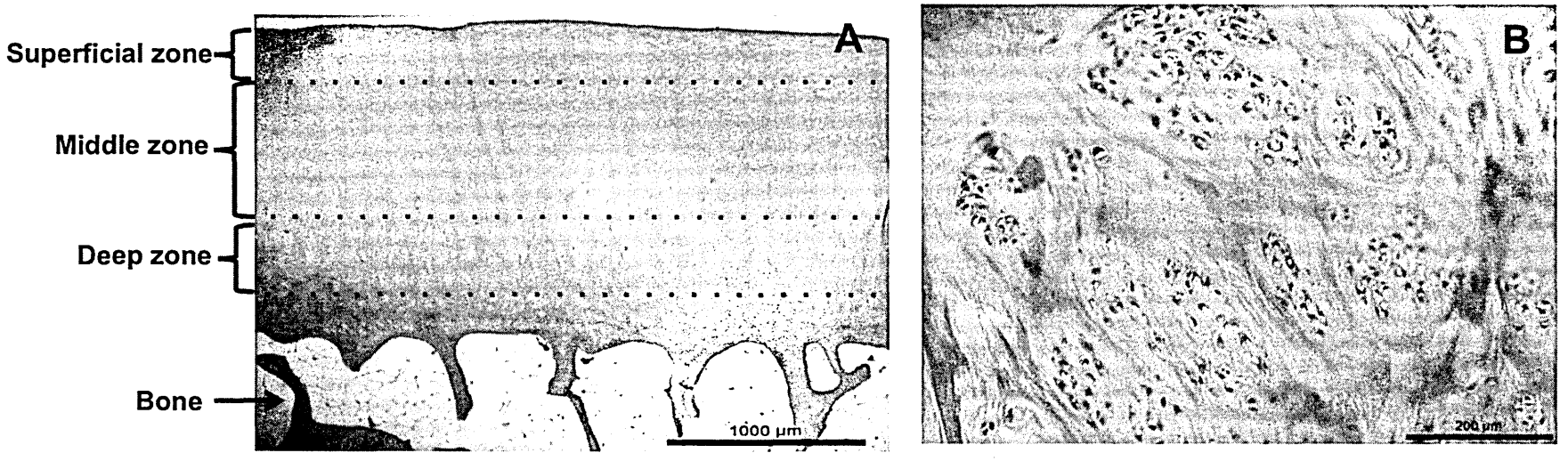

Figure 5.5 IHC Cell Counting Zones. (A) Cells were counted in the superficial zone, middle zone, deep zone and the bone from left to right. **(B)** Cells in clusters of more than 4 were counted where present in tissue sections. 200 cells were counted in each of the cartilage zones, clusters and osteocytes in the bone, cells still remaining in the field of view when the 200 cell count had been reached were also included in the total count. Cells in the periphery of the tissue or those present near folds in the tissue were excluded from the count.

5.4.18 Human OA Cartilage Samples

Primary Human chondrocytes were obtained from the articular cartilage removed from patients with symptomatic OA at the time of total knee replacement as described in section 2.4.1.

5.4.19 Macroscopic Grading of Cartilage Tissue

Cartilage tissue was macroscopically graded 0-4 using the Outerbridge Classification at time of surgery prior to isolation of chondrocytes (Cameron *et al,* 2003) as described in section 2.4.2

5.4.20 Isolation of Human Chondrocytes

Human chondrocytes were isolated from cartilage as described in section 2.4.3.

5.4.21 OA Patient Samples

Chondrocytes cultures were derived from OA patient samples of macroscopic grades 2 or 3 (Table 5.5). Full patient sample information can be found in Appendix 1.

5.4.22 Culture of OA Chondrocytes for Cannabinoid Receptor Immunocytochemistry

Chondrocytes were cultured in monolayer until 80% confluent at passage 2. Following trypsinisation as outlined in section 2.4.5 cells were centrifuged at 400g for 10 minutes and resuspended in complete media. Cells were counted using trypan blue exclusion using the Countess cell counter (Invitrogen). Chondrocytes were seeded at $1x10^5$ cells per well in 8 well chamber slides in complete media. Cells were allowed to adhere overnight at 37°C in a humidified atmosphere of 5% CO_2 prior to WIN-55 treatment.

Analysis Performed	Monolayer Culture	
	Grade 2	Grade 3
Cannabinoid Receptor Immunocytochemistry	HC21(4), HC23(4)	HC5(1), HC3(4),
PPARα, δ and γ mRNA expression	HC13(1), HC23(4)	HC15(4), HC11(3), HC16(4)

Table 5.5 The patient samples used for each analysis performed on chondrocytes from different macroscopic grades of OA cartilage. Full details of samples used in these investigations can be found in Appendix 1.

5.4.23 WIN-55 treatment of OA Chondrocytes for Cannabinoid Receptor Immunocytochemistry

Cells were washed twice in 1xPBS and the complete media replaced with 200 µl serum free media+BSA per well supplemented with 10 µM WIN-55 and incubated at 37°C for 48 hours. 0.1% DMSO was used as a vehicle control at the same concentration present in 10 µM WIN-55.

5.4.24 Cannabinoid Receptor Immunocytochemistry

Culture media was removed and cells washed twice in 1xPBS. Cells were fixed in 4% formalin v/v in 1xPBS at room temperature for 30 minutes. Chambers were removed from slides and cells were permeablised and non-specific binding sites were blocked as outlined in section 5.4.12. Blocking solution was removed and primary antibodies were diluted in 1% w/v BSA in 1xTBS (Table 5.4) and 100 µl of primary antibody or IgG control was applied to each section and incubated overnight at 4°C. Cells were washed and secondary antibody was applied and detected as described in section 5.4.13. Slides were cleared, dehydrated and mounted as outlined in section 5.4.3.

5.4.25 Immunocytochemistry Visualisation

Cells were visualised and images captured as outlined in section 5.4.15.

5.4.26 WIN-55 and IL-1β Treatment of OA Chondrocytes Cultured in Monolayer for Real-time PCR Analysis.

Cells were cultured in monolayer until 80% confluent before passaging as described in section 2.3.5. Chondrocytes were seeded in 6 well culture plates at a cell density of $5x10^5$ as described in section 2.4.6. Cell treatments were performed as outlined in section 2.4.7. Treatments were performed in triplicate on chondrocytes isolated from macroscopic grade 2 or grade 3 cartilage (Table 5.1).

5.4.27 RNA Extraction from Cells Cultured in Monolayer

Isolation of RNA was performed as described in section 2.4.13.

5.4.28 Reverse Transcription and Real-time PCR

RNA was reversed transcribed to cDNA as described in section 2.3.16. Taqman PCR was performed on cDNA as described in section 2.4.18. using pre-designed Taqman Gene Expression Assays (Table 5.6; Life Technologies).

5.4.29 Real-time PCR Analysis

The data obtained from chondrocytes isolated from grade 2 and 3 cartilage were combined prior to analysis (Table 5.1). Real-time PCR data was analysed using the $2^{-\Delta\Delta CT}$ (Livak and Schmittgen 2001) as described in section 2.4.20.

Taqman Gene Expression Assay	Assay ID
PPARα	Hs00947539_m1
PPARδ	Hs00606407_m1
PPARγ	Hs01115513_m1

Table 5.6 Taqman Gene Expression IDs

5.4.30 Statistical Analysis

Statistical analysis was performed as described in section 2.5.

5.5 Results

5.5.1 Cannabinoid Receptor Expression in Cartilage and Bone

5.5.1.1 CB1 Expression

CB1 receptor expression was observed in all cartilage and bone samples. Positive staining was observed in the cytoplasm and nucleus of the chondrocytes in all the zones of the cartilage and the osteocytes in the bone (Figure 5.6).

There was no significant difference between CB1 immunopositivity in chondrocytes in the superficial zone, middle zone, deep zone, clusters or osteocytes in the bone with grade of degeneration (Figure 5.7A-E). There was a significant decrease in CB1 immunopositivity in osteocytes compared to CB1 expression in chondrocytes in the superficial zone ($p<0.01$), middle zone ($p<0.001$), deep zone ($p<0.001$) and clusters ($p<0.001$) (Figure 5.7F). However, there was no significant difference in CB1 percentage immunopositivity between the superficial zone, middle zone, deep zone or clusters ($p>0.05$) (Figure 5.7F).

Regression analysis of CB1 immunopositivity and the microscopic grade of degeneration confirmed that there was no relationship between CB1 expression and grade of degeneration in the chondrocytes of the cartilage in the superficial zone, middle zone, deep zone or clusters or the osteocytes in the bone (Data not shown).

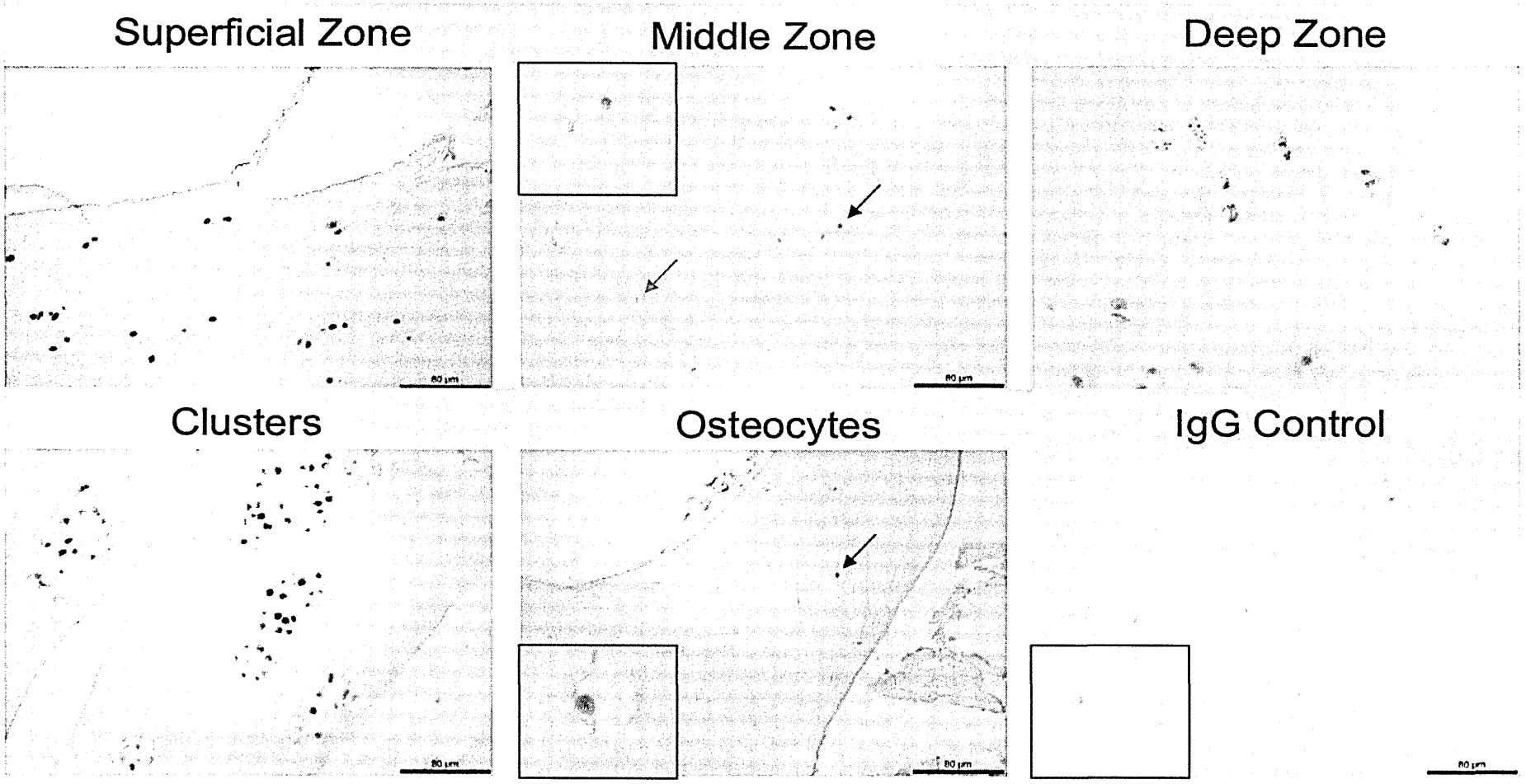

Figure 5.6 CB1 expression and localisation in OA cartilage and osteocytes. Images show CB1 positivity in the superficial zone (HC18(1)), middle zone (HC17(1)), deep zone (HC19(1)), clusters (HC17(6)) and osteocytes (HC18(1)). Positivity is indicated by the presence of brown staining (black arrow). Tissue was counterstained with Mayer's Haematoxylin for the identification of negative cells (red arrow).

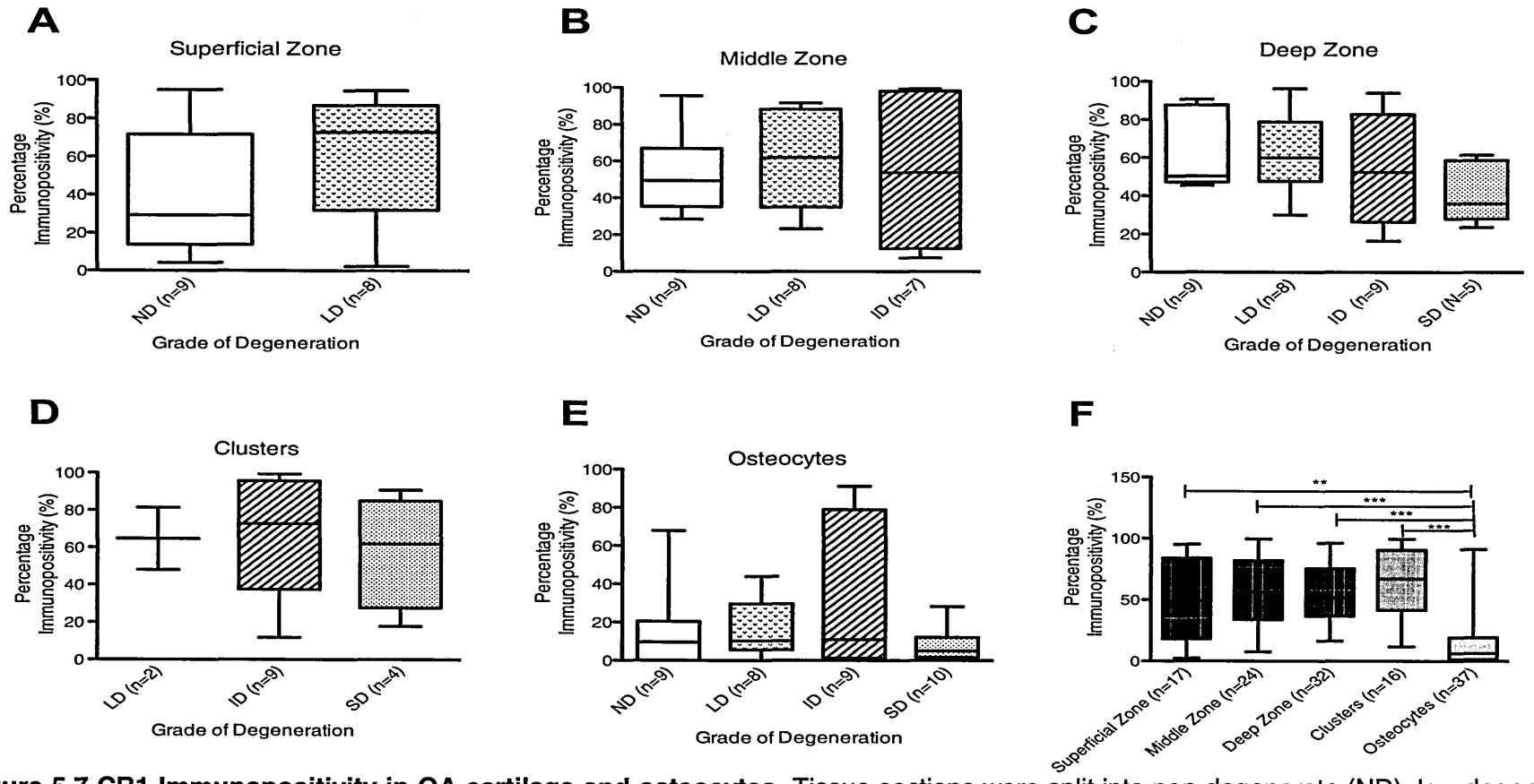

Figure 5.7 CB1 Immunopositivity in OA cartilage and osteocytes. Tissue sections were split into non-degenerate (ND), low degeneration, (LD), intermediate degeneration (ID) and severe degeneration (SD) study groups. Immunopositivity was determined in chondrocytes of the superficial zone **(A),** middle zone **(B),** deep zone **(C)** and clusters **(D)** and the osteocytes of the bone **(E)** within the different study groups Combination of cartilage zones immunopositivity **(F).** For each zone, 200 chondrocytes (cartilage) or osteocytes (bone) were counted and the number of positive cells present in each was expressed as a percentage. ***p<0.001 and **p<0.01. (NB; superficial zone only present in ND and LD cartilage and middle zone only present in ND, LD and ID cartilage).

5.5.1.2 CB2 Expression

CB2 receptor expression was observed in all cartilage samples. Positive staining was observed in the cytoplasm and nucleus of the chondrocytes in all the zones of the cartilage including the osteocytes in the bone (Figure 5.8). Staining was less intense in the deep zone and osteocytes (Figure 5.9C&E).

There was no significant difference between CB2 immunopositivity in the superficial zone, middle zone, deep zone, clusters or osteocytes with grade of degeneration (Figure 5.9A-E). There was a significant decrease in CB2 immunopositivity in osteocytes compared to CB2 expression in chondrocytes in the superficial zone, middle zone, deep zone and clusters ($p<0.001$) (Figure 5.9F). There was no significant difference in CB2 expression between the superficial zone, middle zone, deep zone or clusters ($p>0.05$) (Figure 5.9F).

Regression analysis of CB2 immunopositivity and the microscopic grade of degeneration confirmed that there was no relationship between CB2 expression and grade of degeneration in the chondrocytes of the cartilage in the superficial zone, middle zone, deep zone or clusters or the osteocytes in the bone (Data not shown).

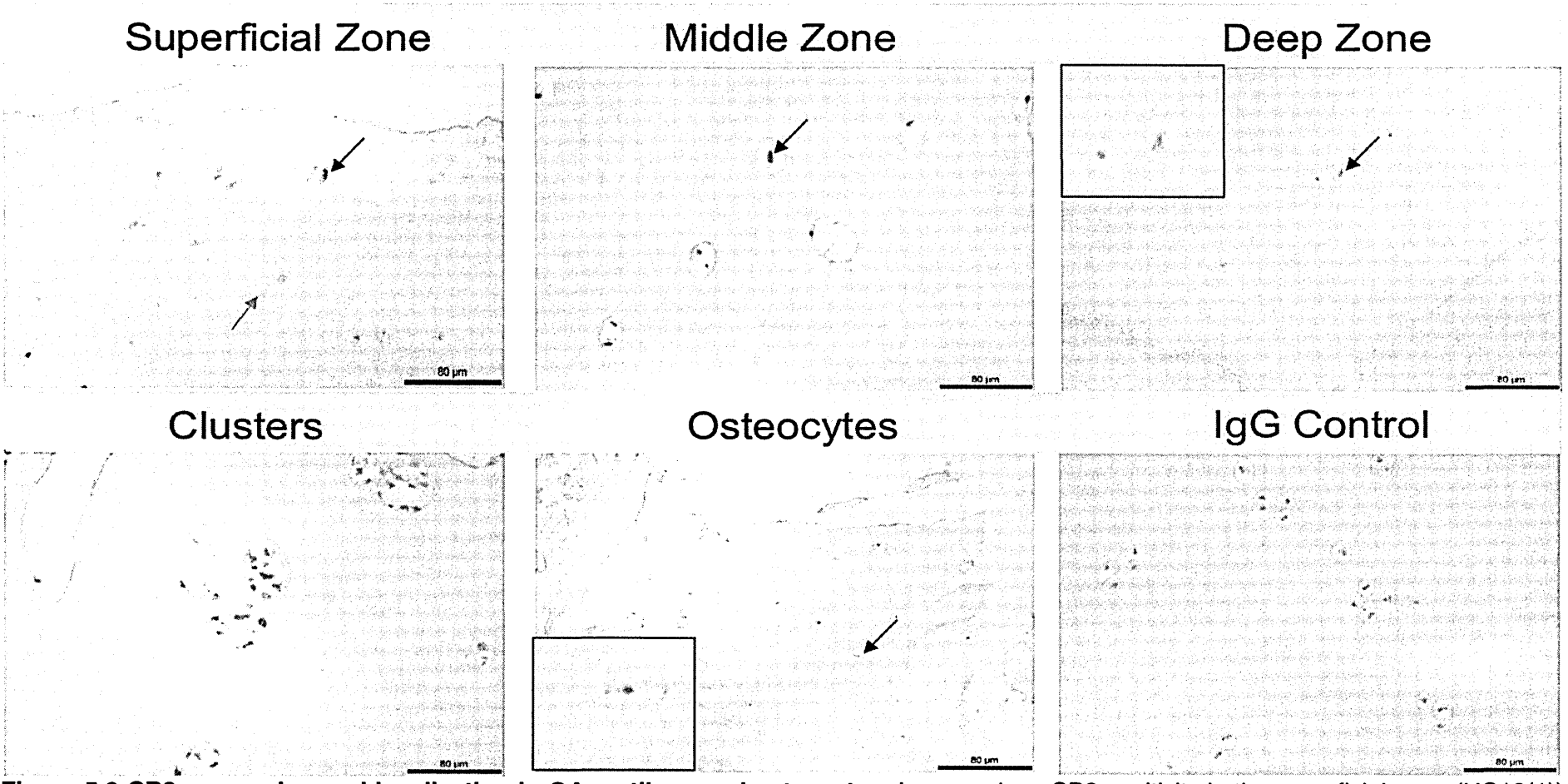

Figure 5.8 CB2 expression and localisation in OA cartilage and osteocytes. Images show CB2 positivity in the superficial zone (HC18(1)) middle zone (HC18(1)), deep zone (HC18(1)), clusters (HC17(6)) and osteocytes (HC18(1)). Positivity is indicated by the presence of brown staining (black arrows). Tissue was counterstained with Mayer's Haematoxylin for the identification of negative cells (red arrow).

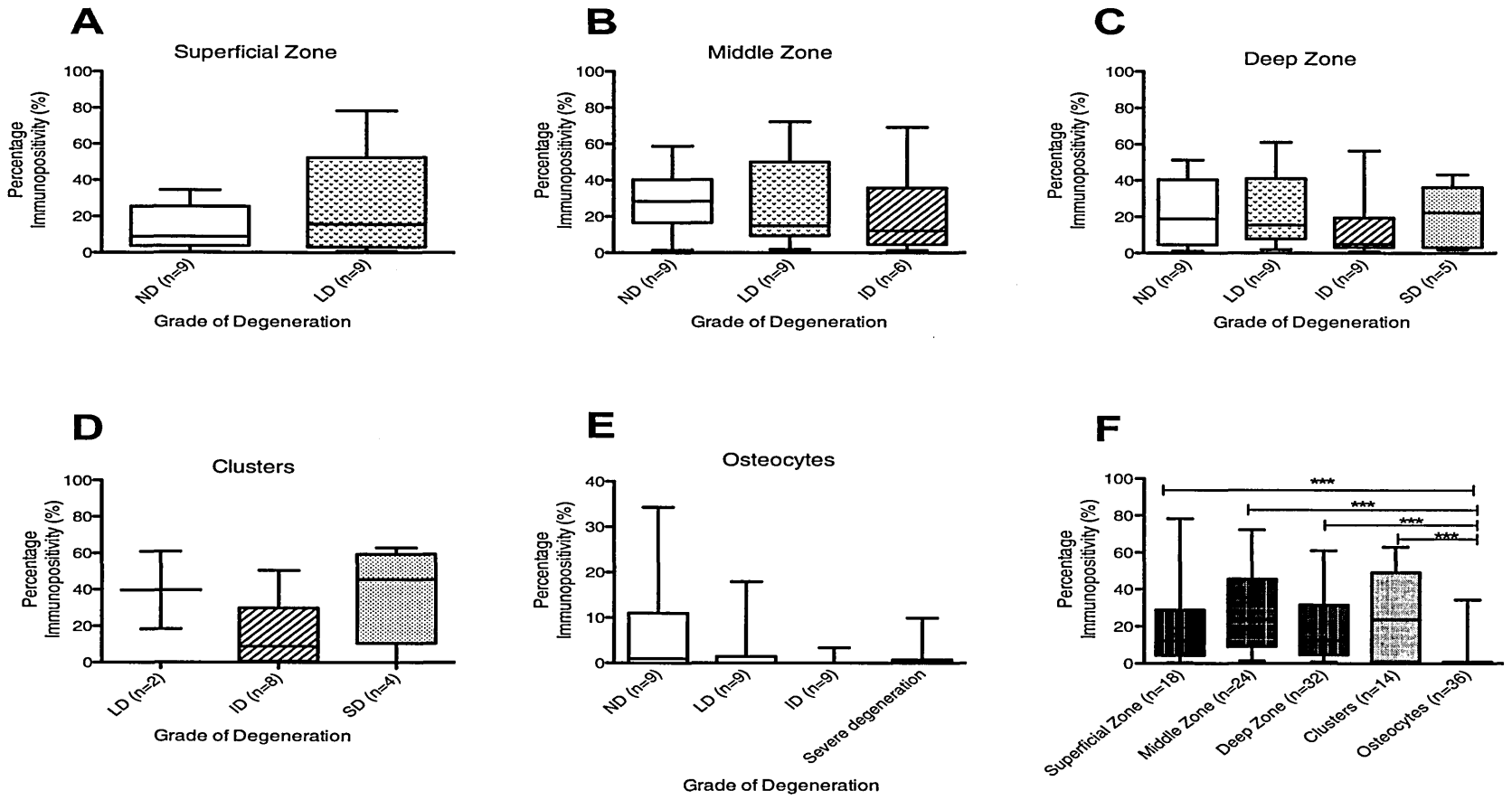

Figure 5.9 CB2 Immunopositivity in OA cartilage and osteocytes. Tissue sections were split into non-degenerate (ND), low degeneration (LD), intermediate degeneration (ID) and severe degeneration (SD) study groups. Immunopositivity was determined in chondrocytes of the **(A)** superficial zone, **(B)** middle zone, **(C)** deep zone and **(D)** clusters and **(E)** the osteocytes of the bone within the different study groups. Combination of cartilage zones immunopositivity **(F)**. For each zone, 200 chondrocytes (cartilage) or osteocytes (bone) were counted and the number of positive cells present in each was expressed as a percentage. ***p<0.001. (NB superficial zone only present in ND and LD cartilage and middle zone only present in ND, LD and ID cartilage).

5.5.1.3 GPR55

GPR55 receptor expression was observed in all cartilage samples. Positive staining was observed in the cytoplasm and nucleus of the chondrocytes in all the zones of the cartilage including the osteocytes in the bone (Figure 5.10).

There was no significant difference between GPR55 immunopositivity in the superficial zone, middle zone, deep zone, clusters or osteocytes with grade of degeneration (Figure 5.11A-E). There was a significant decrease in GPR55 immunopositivity in osteocytes compared to GPR55 expression in chondrocytes in the superficial zone, middle zone, deep zone and clusters ($p<0.001$) (Figure 5.11F). There was no significant difference in GPR55 percentage immunopositivity between the superficial zone, middle zone, deep zone or clusters ($p>0.05$).

Regression analysis of GPR55 immunopositivity and the microscopic grade of degeneration confirmed that there was no relationship between GPR55 expression and grade of degeneration in the chondrocytes of the cartilage in the superficial zone, middle zone, deep zone or clusters or the osteocytes in the bone (Data not shown).

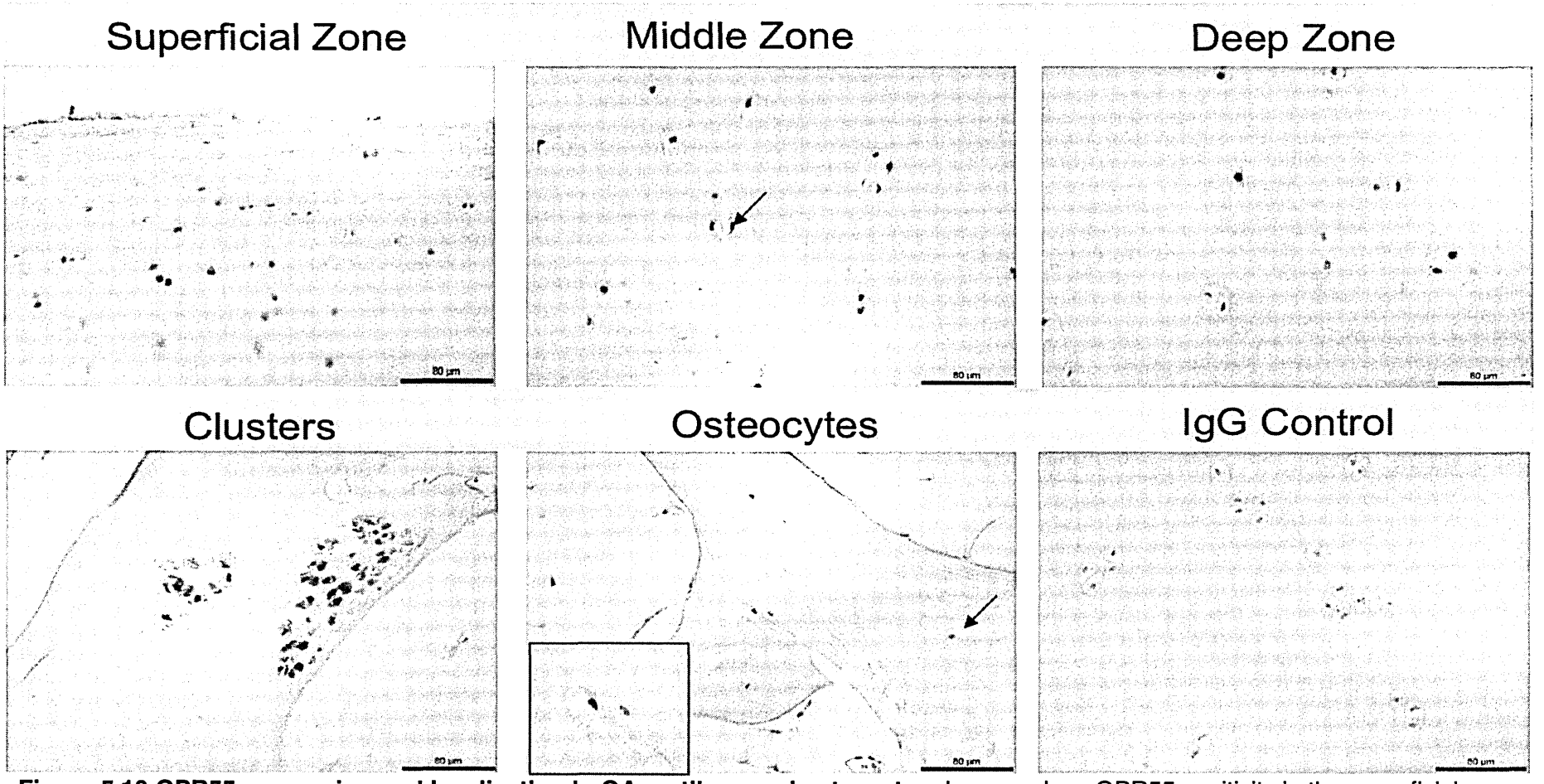

Figure 5.10 GPR55 expression and localisation in OA cartilage and osteocytes. Images show GPR55 positivity in the superficial zone (HC19(1)) middle zone (HC19(1)), deep zone (HC19(1)), clusters (HC17(6)) and osteocytes (HC19(1)). Positivity is indicated by the presence of brown staining (black arrows). Tissue was counterstained with Mayer's Haematoxylin for the identification of negative cells.

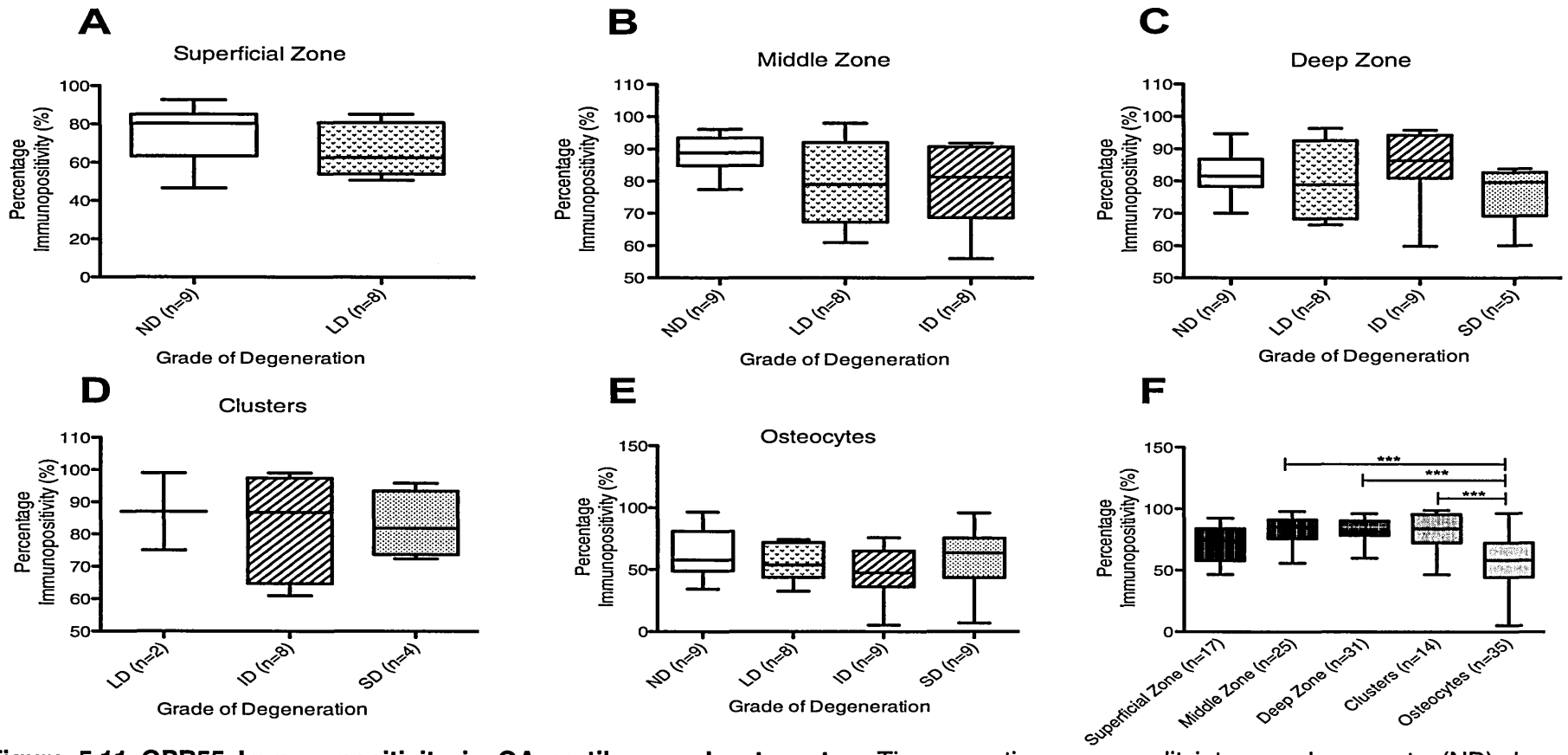

Figure 5.11 GPR55 Immunopositivity in OA cartilage and osteocytes. Tissue sections were split into non-degenerate (ND), low degeneration (LD), intermediate degeneration (ID) and severe degeneration (SD) study groups. Immunopositivity was determined in chondrocytes of the superficial zone **(A),** middle zone **(B)**, deep zone **(C)** and clusters **(D)** and the osteocytes **(E)** of the bone within the different study groups. Combination of cartilage zones immunopositivity **(F)**. For each zone, 200 chondrocytes (cartilage) or osteocytes (bone) were counted and the number of positive cells present in each was expressed as a percentage. ***$p<0.001$ (NB superficial zone only present in ND and LD cartilage and middle zone only present in ND, LD and ID cartilage).

5.5.1.4 GPR18 Expression

GPR18 receptor expression was observed in all cartilage samples. Positive staining was observed in the cytoplasm and nucleus of the chondrocytes in all the zones of the cartilage (Figure 5.12). GPR18 receptor expression in osteocytes was observed in 10 out of the 34 samples (Figure 5.12).

There was no significant difference between GPR18 immunopositivity in the superficial zone, middle zone, clusters or osteocytes with grade of degeneration (Figure 5.13A,B,D,E). However in the deep zone of the cartilage there was a significant decrease in GPR18 immunopositivity in the severe degeneration samples compared to the low degeneration samples ($p<0.05$) (Figure 5.13C). Increased immunopositivity of GPR18 was detected in the middle ($p<0.001$) and deep zone ($p<0.05$) compared to the superficial zone (Figure 5.13F).

Regression analysis of GPR18 immunopositivity and the microscopic grade of degeneration confirmed that there was a relationship between GPR18 expression and grade of degeneration in the chondrocytes in the deep zone of the cartilage (Figure 5.14). However, there was no relationship between GPR18 expression and grade of degeneration in the chondrocytes of the superficial zone, middle zone or clusters, or the osteocytes in the bone (Data not shown).

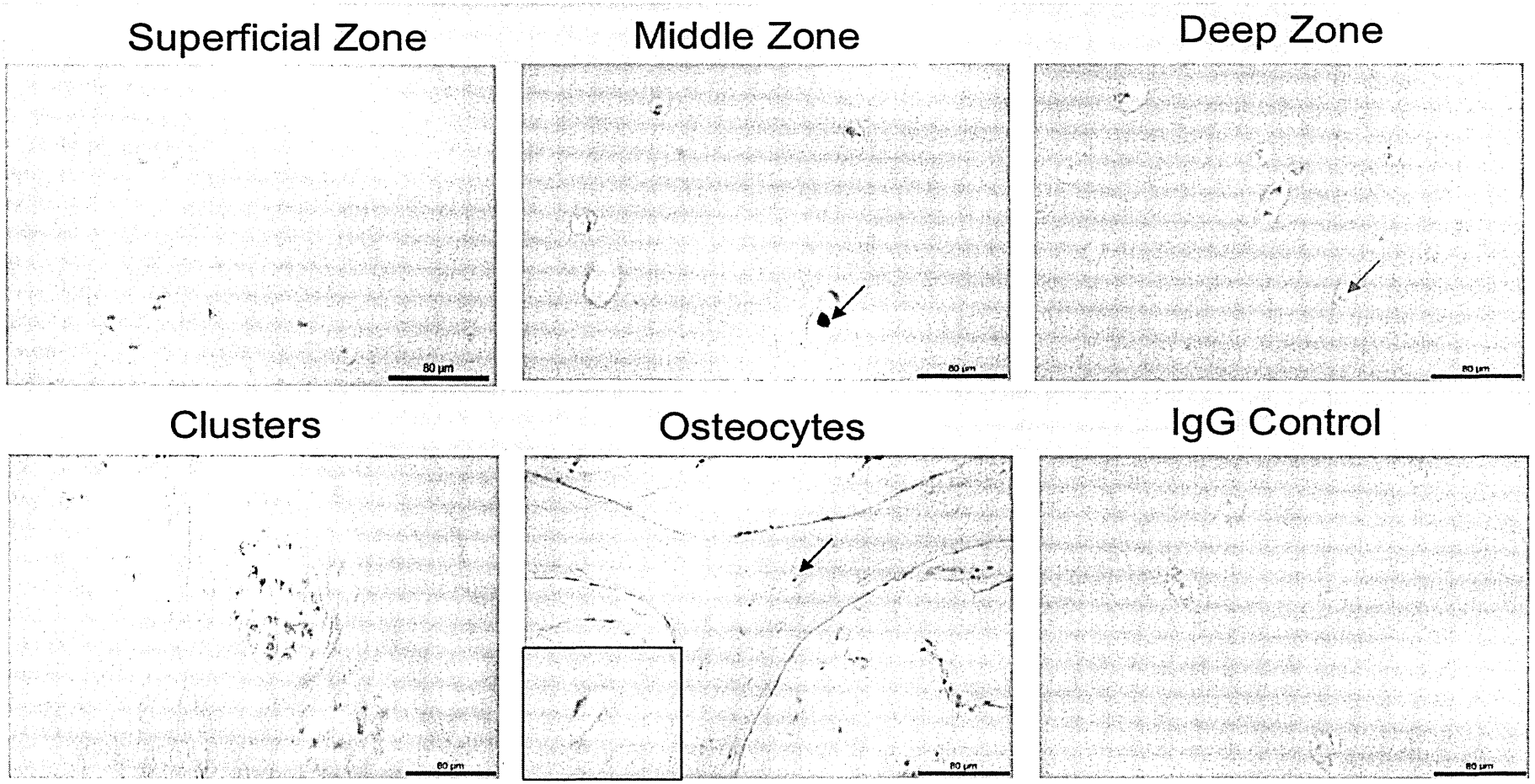

Figure 5.12 GPR18 expression and localisation in OA cartilage and osteocytes. Images show GPR18 positivity in the superficial zone (HC17(1)) middle zone (HC4(1)), deep zone (HC4(1)), clusters (HC2(3)) and osteocytes (HC9(7)). Positivity is indicated by the presence of brown staining (black arrows). Tissue was counterstained with Mayer's Haematoxylin for the identification of negative cells (red arrow).

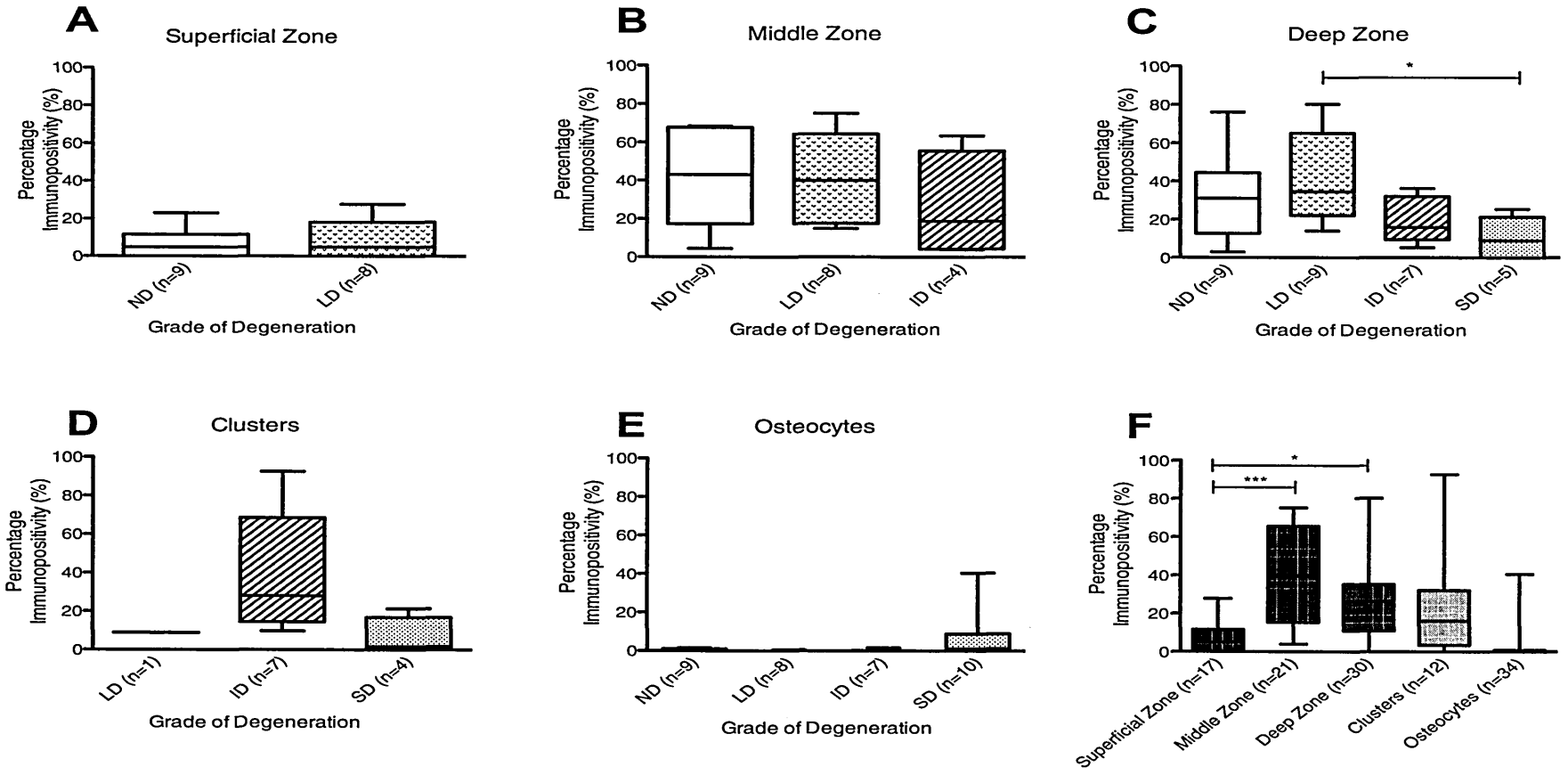

Figure 5.13 GPR18 Immunopositivity in OA cartilage and osteocytes. Tissue sections were split into non-degenerate (ND) low degeneration (LD), intermediate degeneration (ID) and severe degeneration (SD) study groups. Immunopositivity was determined in chondrocytes of the superficial zone **(A),** middle zone **(B)**, deep zone **(C)** and clusters **(D)** and the osteocytes **(E)** of the bone within the different study groups. Combination of cartilage zones immunopositivity **(F)**. For each zone, 200 chondrocytes (cartilage) or osteocytes (bone) were counted and the number of positive cells present in each was expressed as a percentage. *p<0.05, ***p<0.001 (NB superficial zone only present in ND and LD cartilage and middle zone only present in ND, LD and ID cartilage).

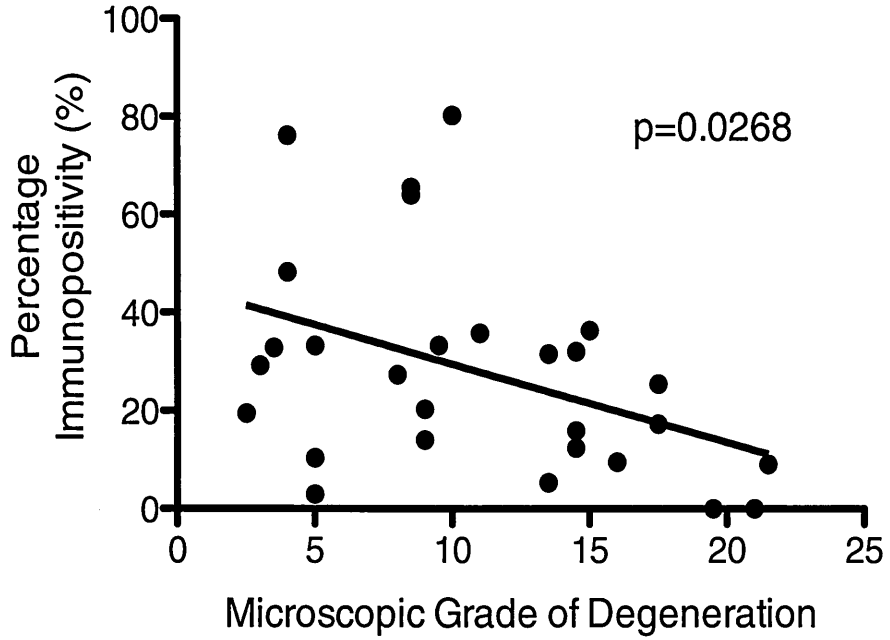

Figure 5.14 GPR18 immunopositivity in the deep zone of cartilage. Regression analysis of GPR18 percentage immunopositivity against microscopic grade of degeneration.

5.5.1.5 TRPV1 Expression

TRPV1 receptor expression was observed in all cartilage samples. Positive staining was observed in the cytoplasm and nucleus of the chondrocytes in all the zones of the cartilage (Figure 5.15). TRPV1 receptor expression in osteocytes was observed in all but three samples (two low-degenerate and one intermediate).

There was no significant difference between TRPV1 immunopositivity in the superficial zone, middle zone, clusters or osteocytes with grade of degeneration (Figure 5.16 A,B,D,E). However in the deep zone of the cartilage there was a significant decrease in TRPV1 immunopositivity in the severe degeneration samples compared to the low degeneration samples ($p<0.05$) (Figure 5.16C). There was a significant decrease in TRPV1 immunopositivity in osteocytes compared to TRPV1 expression in chondrocytes of the superficial zone, middle zone, deep zone and clusters ($p<0.001$) (Figure 5.16F). There was no significant difference in TRPV1 percentage immunopositivity between the superficial zone, middle zone, deep zone or clusters ($p>0.05$).

Regression analysis of TRPV1 immunopositivity and the microscopic grade of degeneration demonstrated that there was no relationship between TRPV1 expression and grade of degeneration in the chondrocytes of the cartilage in the superficial zone, middle zone, deep zone or clusters, or the osteocytes in the bone (Data not shown).

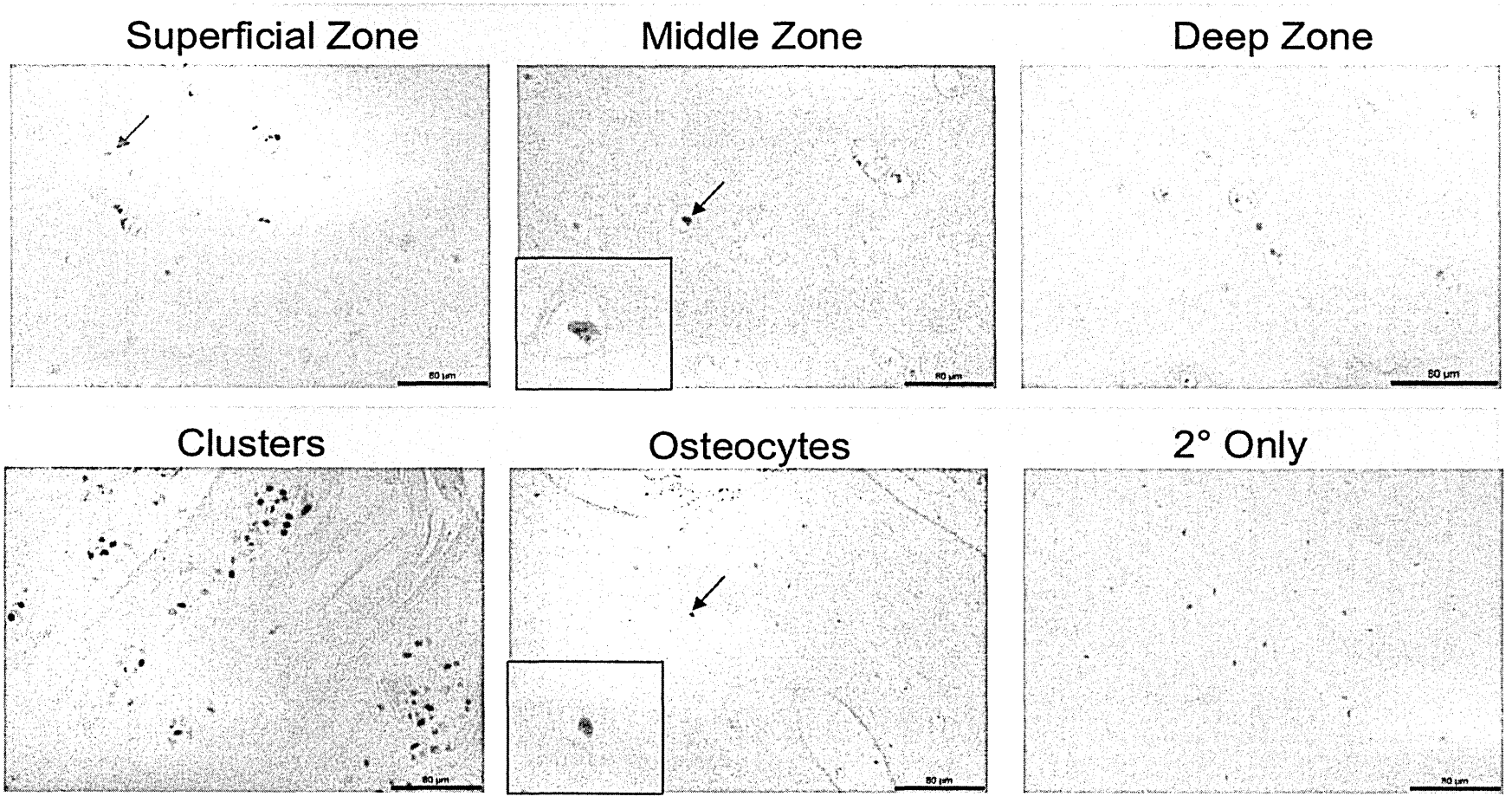

Figure 5.15 TRPV1 expression and localisation in OA cartilage and osteocytes. Images show TRPV1 positivity in the superficial zone (HC17(1)) middle zone (HC4(1)), deep zone (HC4(1)), clusters (HC2(3)) and osteocytes (HC9(7)). Positivity is indicated by the presence of brown staining (black arrows). Tissue was counterstained with Mayer's Haematoxylin for the identification of negative cells (red arrow).

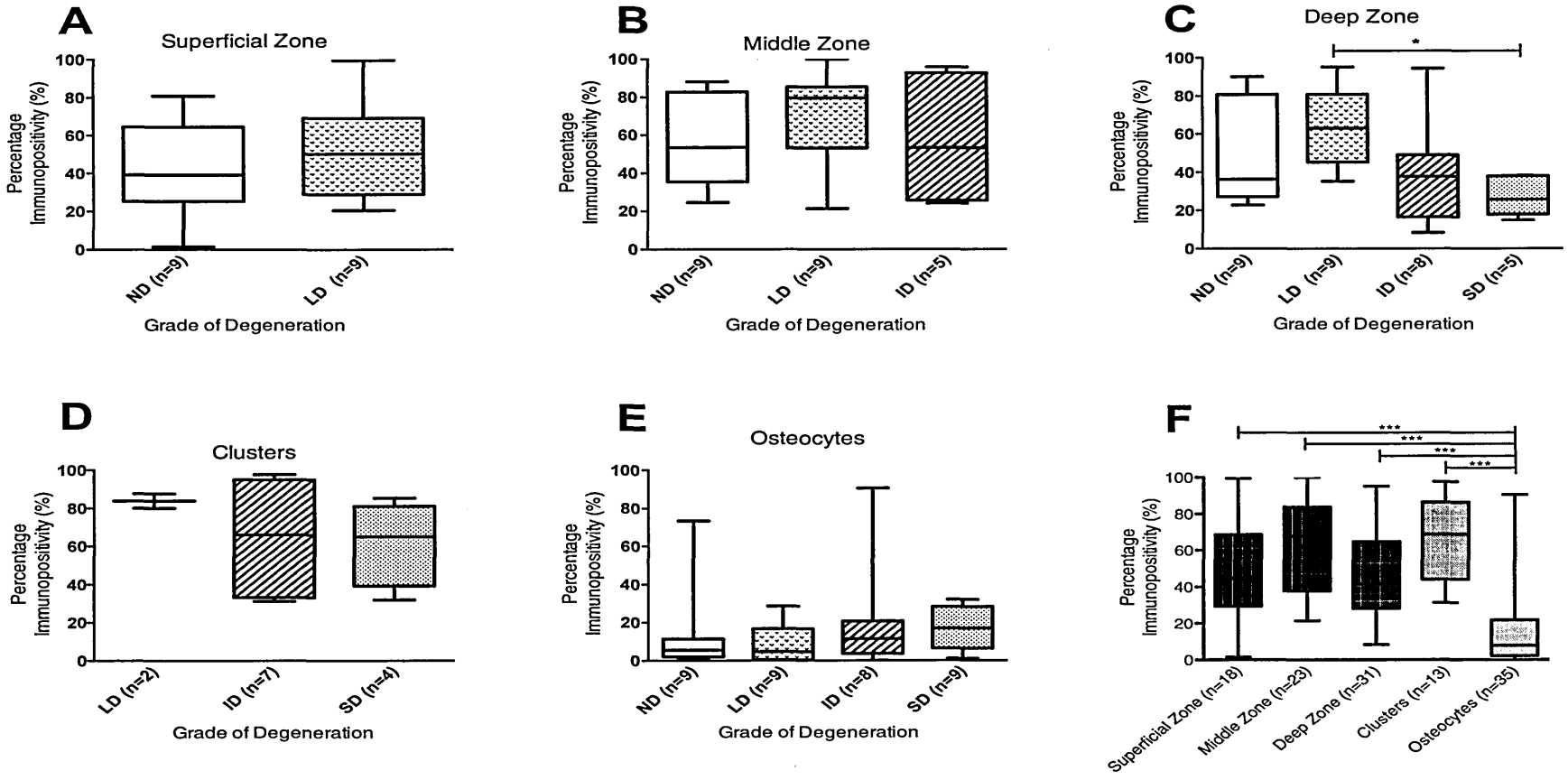

Figure 5.16 TRPV1 Immunopositivity in OA cartilage and bone tissue. Tissue sections were split into non-degenerate (ND), low degeneration (LD), intermediate degeneration (ID) and severe degeneration (SD) study groups. Immunopositivity was determined in chondrocytes of the superficial zone **(A),** middle zone **(B)**, deep zone **(C)** and clusters **(D)** and the osteocytes **(E)** of the bone within the different study groups. Combination of cartilage zones immunopositivity **(F)**. For each zone, 200 chondrocytes (cartilage) or osteocytes (bone) were counted and the number of positive cells present in each was expressed as a percentage. *p<0.05, ***p<0.001. (NB superficial zone only present in ND and LD cartilage and middle zone only present in ND, LD and ID cartilage).

5.5.1.6 PPARα

PPARα expression was observed in all cartilage samples. Positive staining was observed in the cytoplasm and nucleus of the chondrocytes in all the zones of the cartilage (Figure 5.17). PPARα expression in osteocytes was observed in all but one intermediate degenerate sample.

There was no significant difference between PPARα immunopositivity in the superficial zone, middle zone, deep zone, clusters or osteocytes with grade of degeneration (Figure 5.18A-E). There was a significant decrease in PPARα immunopositivity in osteocytes compared to PPARα expression in chondrocytes in the superficial zone ($p<0.01$), middle zone, deep zone and clusters ($p<0.001$) (Figure 5.18F). There was no significant difference in PPARα percentage immunopositivity between the superficial zone, middle zone, deep zone or clusters ($p>0.05$).

Regression analysis of PPARα immunopositivity and the microscopic grade of degeneration confirmed that there was no relationship between PPARα expression and grade of degeneration in the chondrocytes of the cartilage in the superficial zone, middle zone, deep zone or clusters or the osteocytes in the bone (Data not shown).

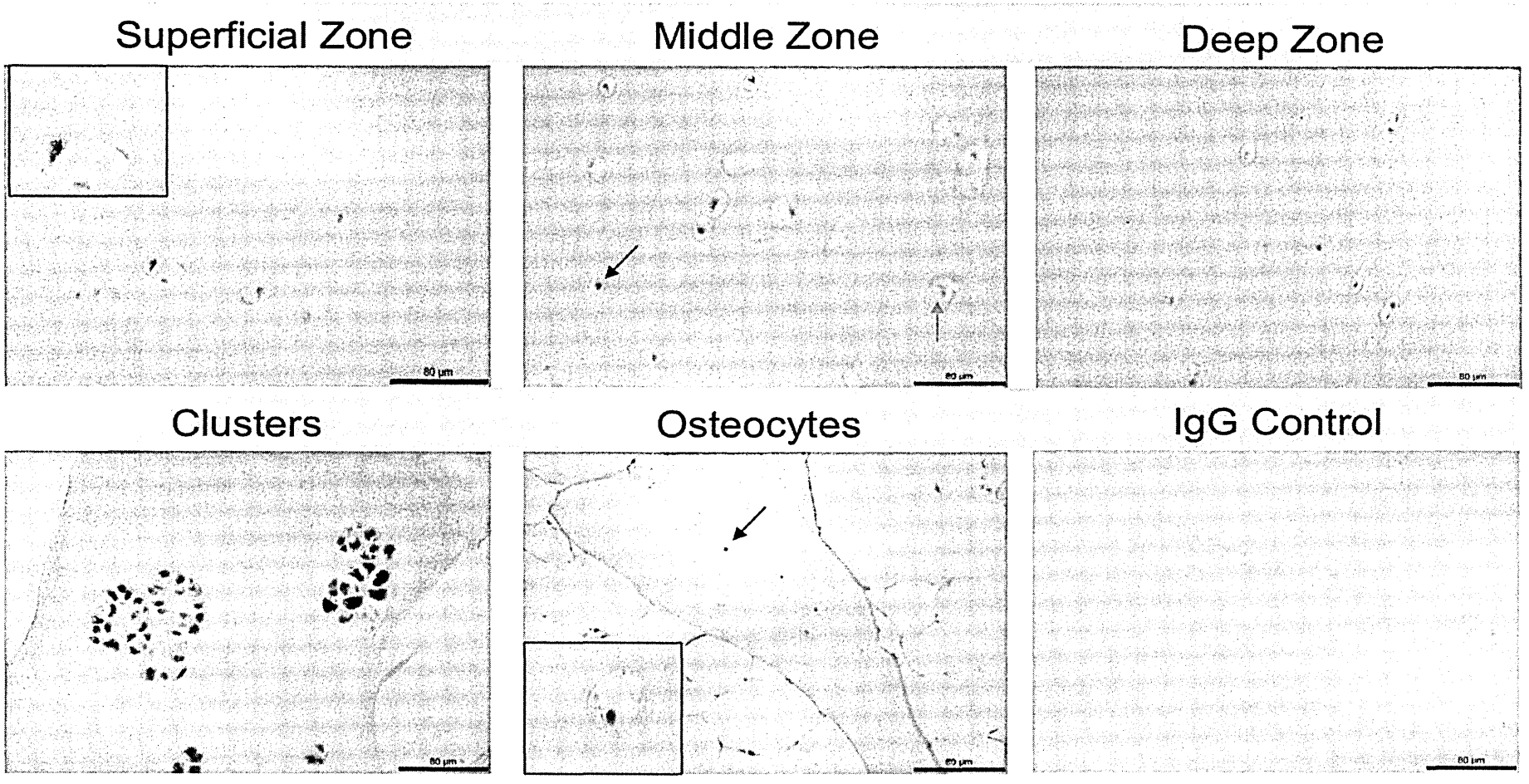

Figure 5.17 PPARα expression and localisation in OA cartilage and osteocytes. Images show PPARα positivity in the superficial zone (HC4(1)) middle zone (HC4(1)), deep zone (HC4(1)), clusters (HC17(6)) and osteocytes (HC17(1)). Positivity is indicated by the presence of brown staining (black arrows). Tissue was counterstained with Mayer's Haematoxylin for the identification of negative cells (red arrow).

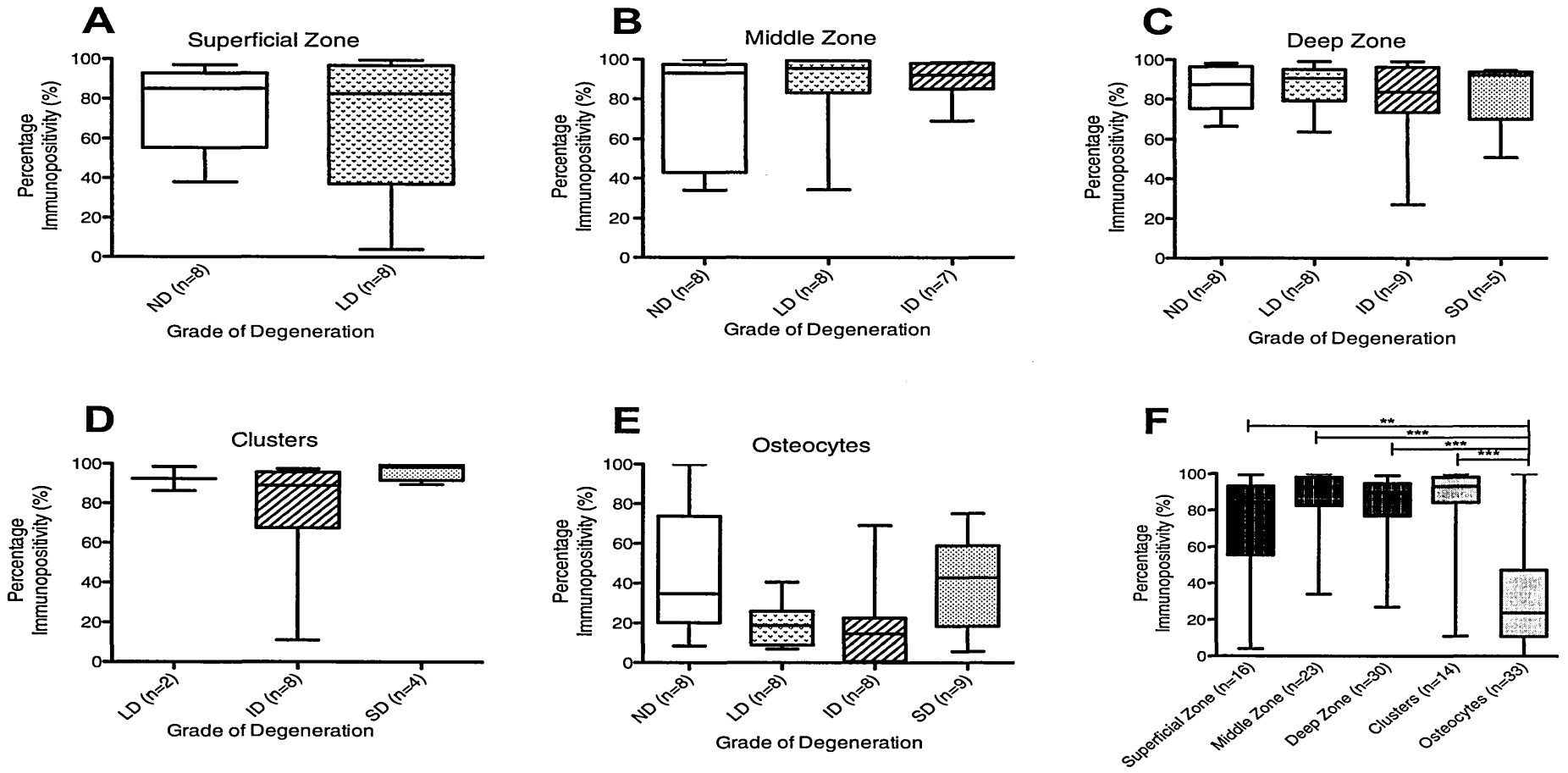

Figure 5.18 PPARα Immunopositivity in OA cartilage and osteocytes. Tissue sections were split into non-degenerate (ND), low degeneration (LD), intermediate degeneration (ID) and severe degeneration (SD) study groups. Immunopositivity was determined in chondrocytes of the superficial zone **(A),** middle zone **(B)**, deep zone **(C)** and clusters **(D)** and the osteocytes **(E)** of the bone within the different study groups. Combination of cartilage zones immunopositivity **(F)**. For each zone, 200 chondrocytes (cartilage) or osteocytes (bone) were counted and the number of positive cells present in each was expressed as a percentage. **p<0.01, ***p<0.001 (NB superficial zone only present in ND and LD cartilage and middle zone only present in ND, LD and ID cartilage).

5.5.1.7 PPARδ

PPARδ expression was observed in all cartilage samples. Positive staining was observed in the cytoplasm and nucleus of the chondrocytes in all the zones of the cartilage and the osteocytes of the bone (Figure 5.19).

There was no significant difference between PPARδ immunopositivity in the superficial zone, middle zone, deep zone, clusters or osteocytes with grade of degeneration (Figure 5.20A-E). There was a significant decrease in PPARδ immunopositivity in osteocytes compared to PPARδ immunopositivity in chondrocytes in the superficial zone, middle zone, deep zone and clusters ($p<0.001$) (Figure 5.20F). There was no significant difference in PPARδ immunopositivity between the superficial zone, middle zone, deep zone or clusters ($p>0.05$).

Regression analysis of PPARδ immunopositivity and the microscopic grade of degeneration confirmed that there was no relationship between PPARδ expression and grade of degeneration in the chondrocytes of the cartilage in the superficial zone, middle zone, deep zone or clusters or the osteocytes in the bone (Data not shown).

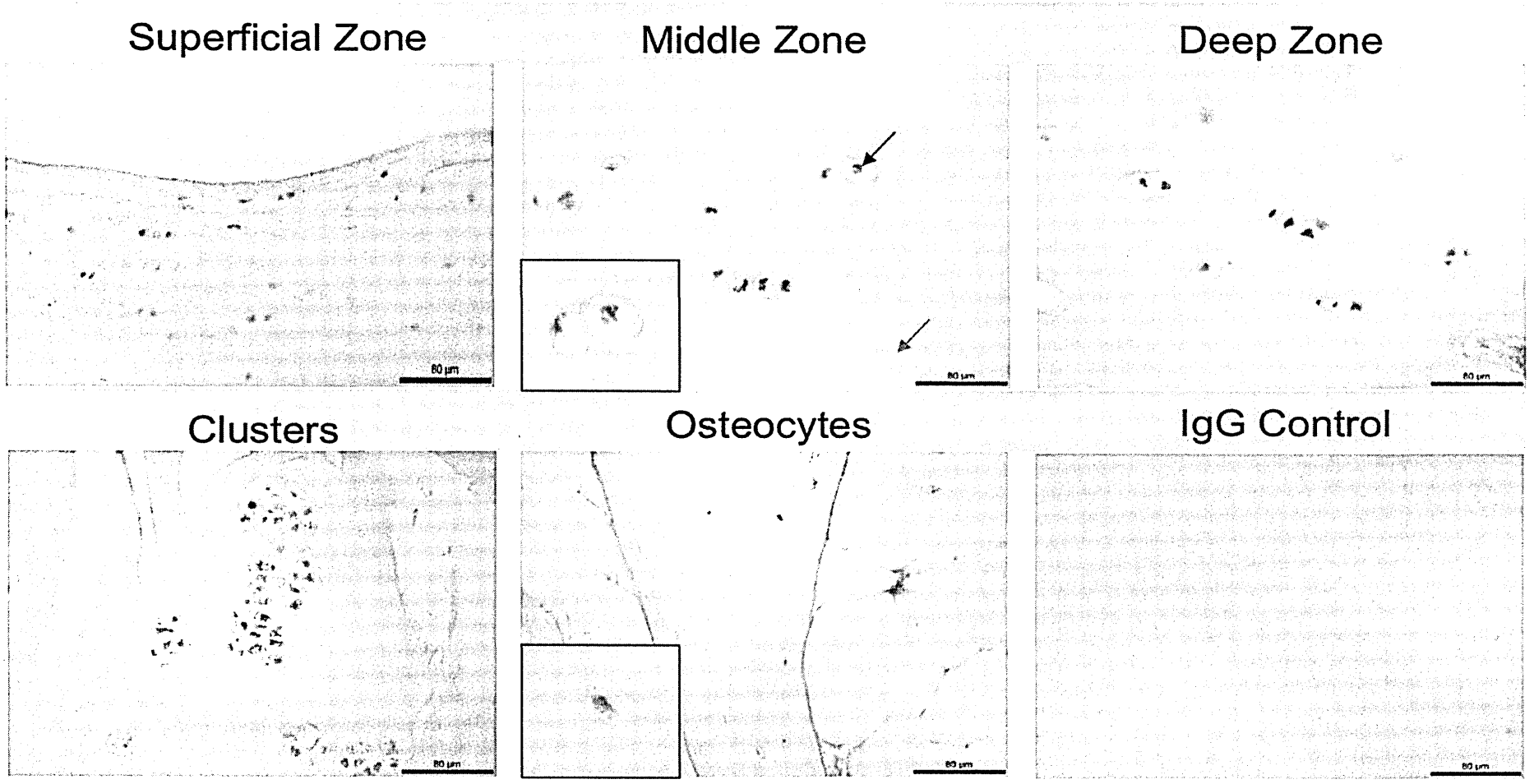

Figure 5.19 PPARδ expression and localisation in OA cartilage and osteocytes. Images show PPARδ positivity in the superficial zone (HC4(1)) middle zone (HC11(2)), deep zone (HC11(2)), clusters (HC17(6)) and osteocytes (HC18(1)). Positivity is indicated by the presence of brown staining (black arrows). Tissue was counterstained with Mayer's Haematoxylin for the identification of negative cells (red arrow).

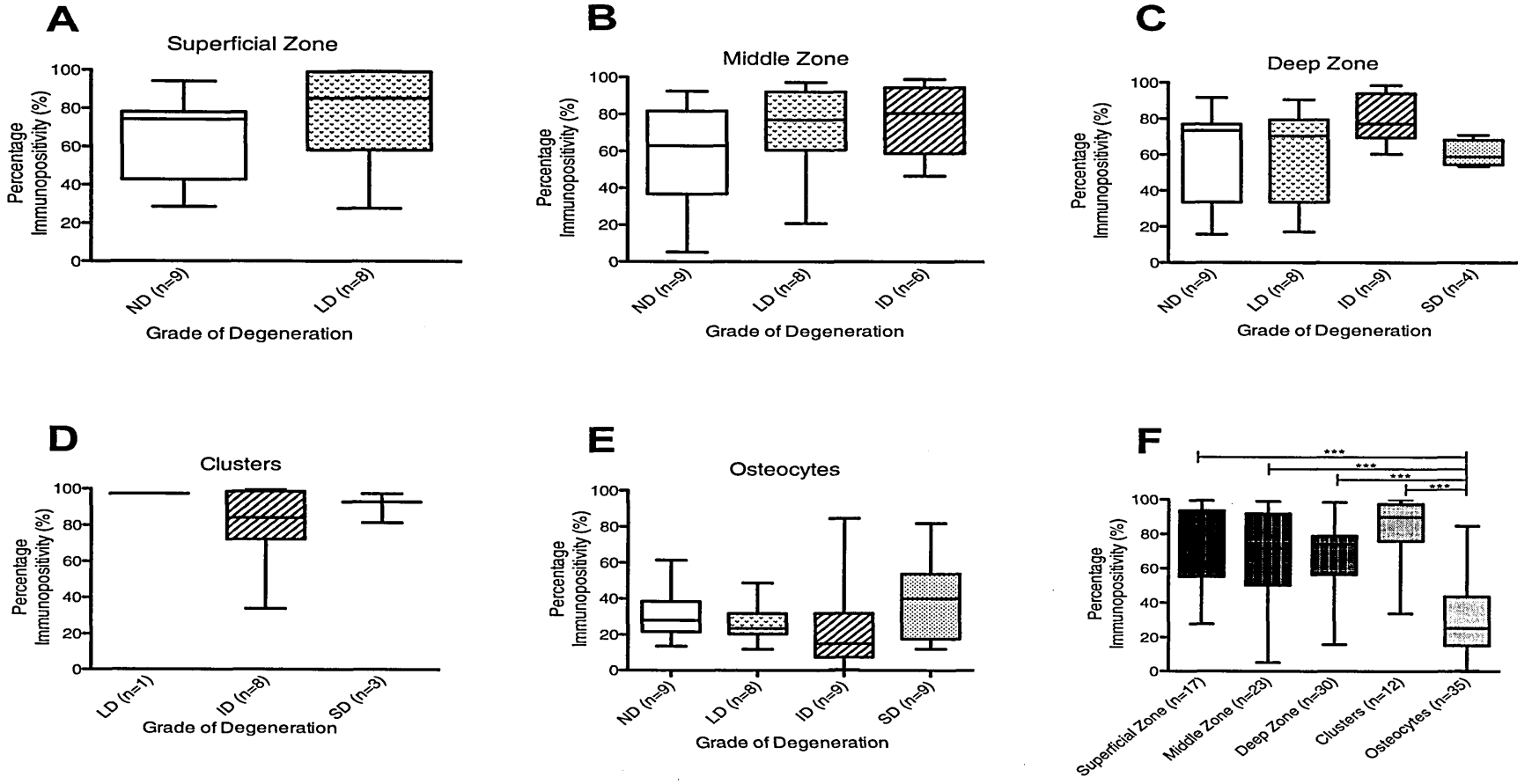

Figure 5.20 PPARδ Immunopositivity in OA cartilage and osteocytes. Tissue sections were split into non-degenerate (ND), low degeneration (LD), intermediate degeneration (ID) and severe degeneration (SD) study groups. Immunopositivity was determined in chondrocytes of the superficial zone **(A),** middle zone **(B)**, deep zone **(C)** and clusters **(D)** and the osteocytes **(E)** of the bone within the different study groups. Combination of cartilage zones immunopositivity **(F)**. For each zone, 200 chondrocytes (cartilage) or osteocytes (bone) were counted and the number of positive cells present in each was expressed as a percentage. ***p<0.001 (NB superficial zone only present in ND and LD cartilage and middle zone only present in ND, LD and ID cartilage).

5.5.1.8 PPARγ

PPARγ expression was observed in all cartilage samples. Positive staining was observed in the cytoplasm and nucleus of the chondrocytes in all the zones of the cartilage and the osteocytes of the bone (Figure 5.21).

There was no significant difference between PPARγ immunopositivity in the superficial zone, middle zone or deep zone with grade of degeneration (Figure 5.22A-C&E). There was a trend towards decrease in PPARγ immunopositivity in the clusters with grade of degeneration; however this was not significant (Figure 5.22D). In the osteocytes there was a significant decrease in PPARγ immunopositivity in the severe degeneration samples compared to non-degenerate ($p<0.05$) (Figure 5.22E). There was a significant decrease in PPARγ immunopositivity in osteocytes compared to PPARγ immunopositivity in chondrocytes in the superficial zone and middle zone ($p<0.001$) (Figure 5.22F). There was no significant difference in PPARγ immunopositivity between the superficial zone, middle zone, deep zone or clusters ($p>0.05$), however PPARγ immunopositivity staining appeared to be more intense in the superficial zone compared to the middle and deep zone (Figure 5.22).

Regression analysis of PPARγ immunopositivity and the microscopic grade of degeneration confirmed that there was a relationship between PPARγ expression and grade of degeneration in the osteocytes (Figure 5.23). However, there was no relationship between PPARγ expression and grade of degeneration in the chondrocytes of the superficial zone, middle zone, deep zone or clusters (Data not shown).

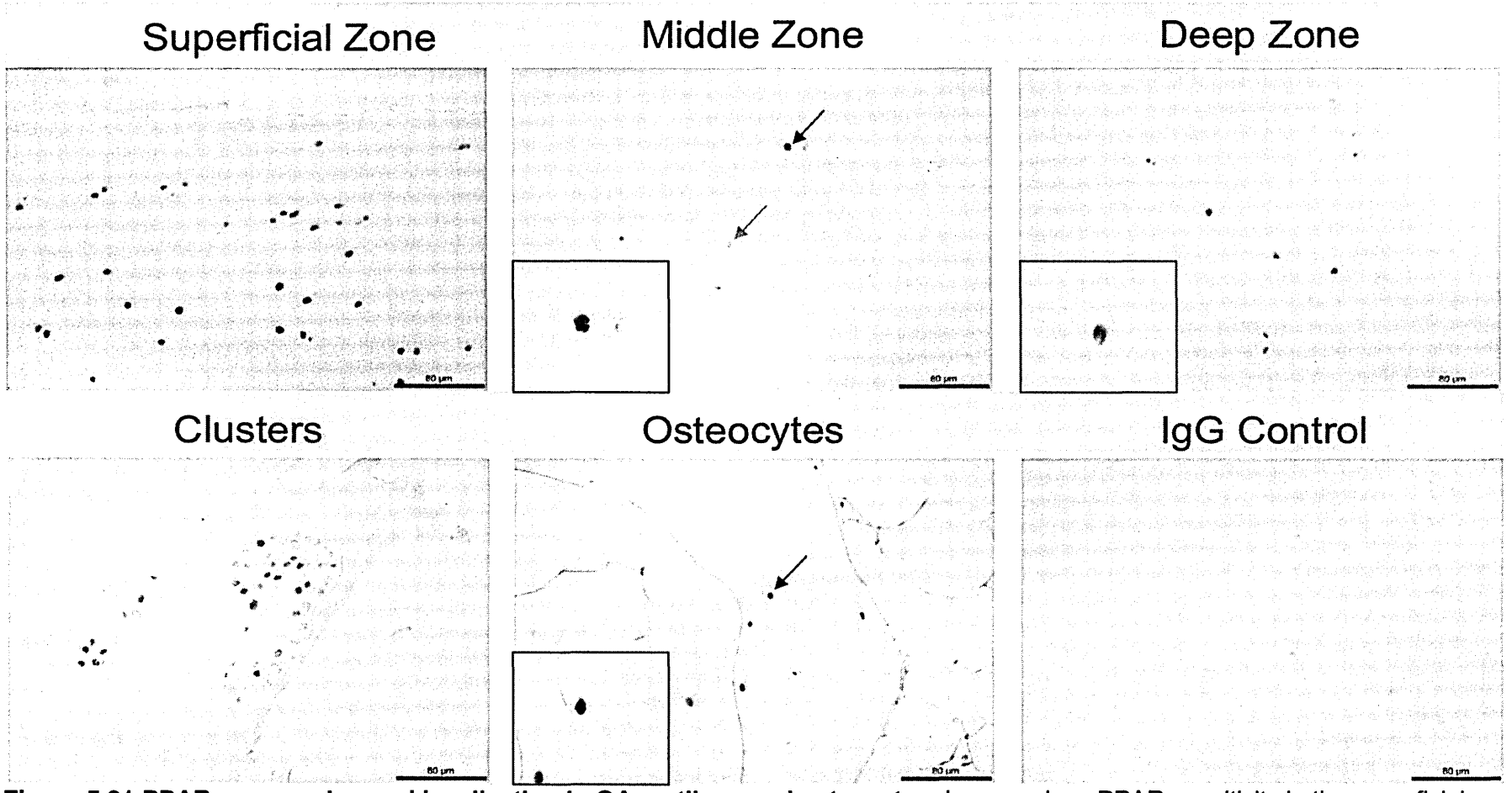

Figure 5.21 PPARγ expression and localisation in OA cartilage and osteocytes. Images show PPARγ positivity in the superficial zone (HC18(1)) middle zone (HC18(1)), deep zone (HC18(1)), clusters (HC17(6)) and osteocytes (HC18(1)). Positivity is indicated by the presence of brown staining (black arrows). Tissue was counterstained with Mayer's Haematoxylin for the identification of negative cells (red arrow).

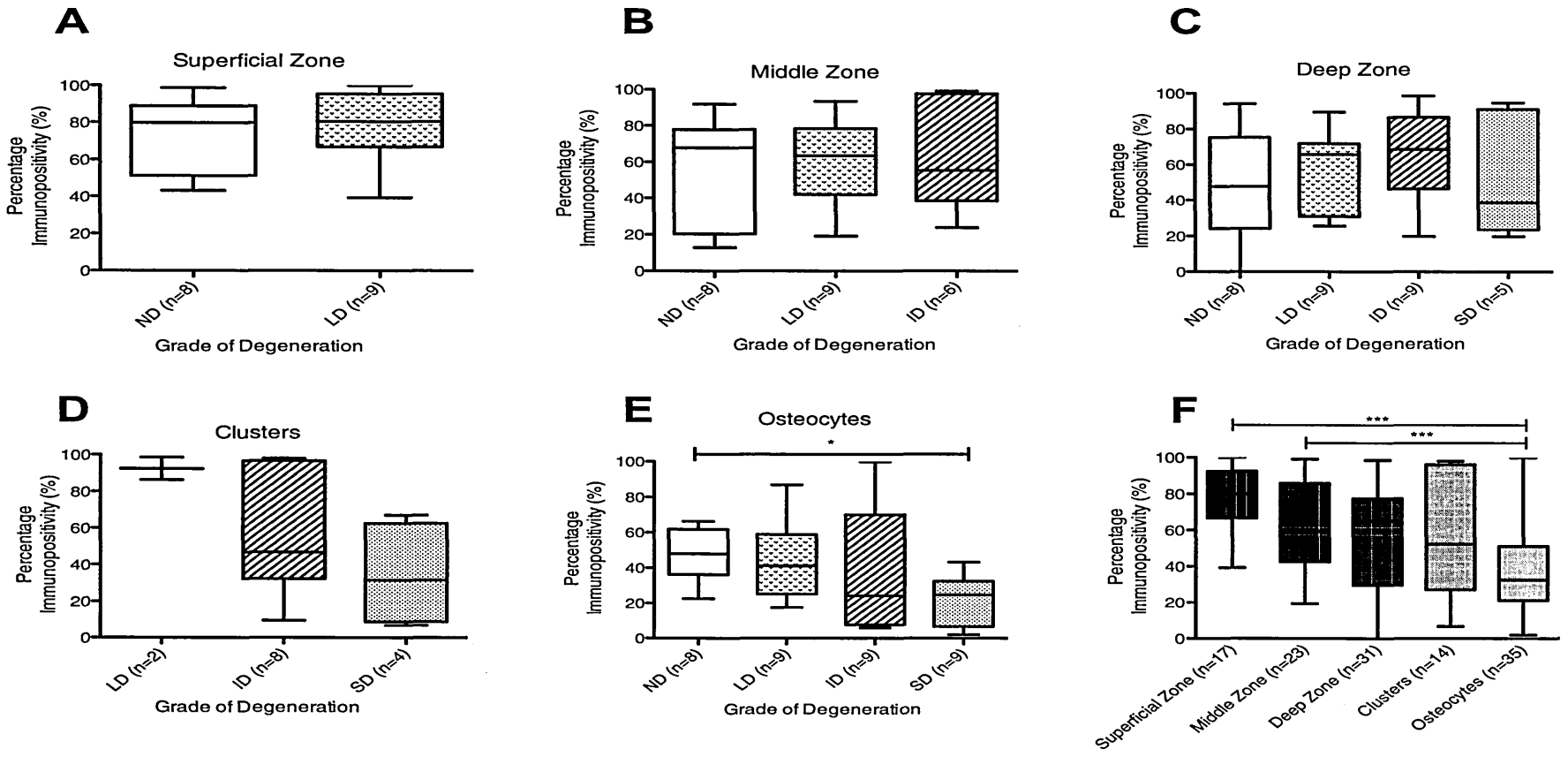

Figure 5.22 PPARγ immunopositivity in OA cartilage and osteocytes. Tissue sections were split into non-degenerate (ND), low degeneration (LD), intermediate degeneration (ID) and severe degeneration (SD) study groups. Immunopositivity was determined in chondrocytes of the superficial zone **(A),** middle zone **(B)**, deep zone **(C)** and clusters **(D)** and the osteocytes **(E)** of the bone within the different study groups. Combination of cartilage zones immunopositivity **(F)**. For each zone, 200 chondrocytes (cartilage) or osteocytes (bone) were counted and the number of positive cells present in each was expressed as a percentage. ***$p<0.001$ *$p<0.05$ (NB superficial zone only present in ND and LD cartilage and middle zone only present in ND, LD and ID cartilage).

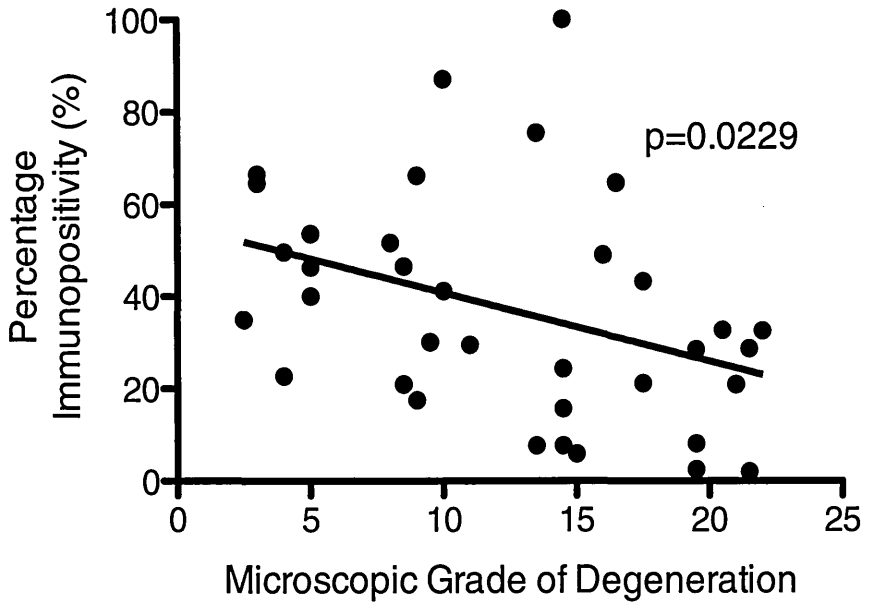

Figure 5.23 PPARγ immunopositivity in osteocytes. Regression analysis of PPARγ percentage immunopositivity against microscopic grade of degeneration.

5.5.2 The Effects of WIN-55 on Cannabinoid Receptor Localisation in OA Chondrocytes

5.5.2.1 CB1 Expression

CB1 expression was localised to the cytoplasm of the chondrocytes in the DMSO control (Figure 5.24A). Following WIN-55 treatment for 48 hours CB1 expression was localised to the nuclei of the chondrocytes (Figure 5.24B). The intensity of CB1 expression was increased following WIN-55 treatment (Figure 5.24B)

5.5.2.2 CB2 Expression

CB2 expression was localised to the cytoplasm of the chondrocytes in the DMSO control (Figure 5.25A). Following WIN-55 treatment for 48 hours CB2 expression was localised to the cytoplasm and nuclei of the chondrocytes (5.25 B).

5.5.2.3 GPR55 Expression

GPR55 expression was localised to the cytoplasm of the chondrocytes in the DMSO control (Figure 5.26A). Following WIN-55 treatment for 48 hours there was no change in GPR55 localisation in the chondrocytes (Figure 5.26B).

5.5.2.4 GPR18 Expression

GPR18 expression was localised to the cytoplasm of the chondrocytes in the DMSO control (Figure 5.27A). Following WIN-55 treatment for 48 hours GPR18 expression was localised to both the nuclei and the cytoplasm of the chondrocytes (Figure 5.27B).

5.5.2.5 TRPV1 Expression

TRPV1 expression was localised to the cytoplasm of the chondrocytes in the DMSO control (Figure 5.28A). Following WIN-55 treatment for 48 hours TRPV1 expression was localised to the nuclei of the chondrocytes (Figure 5.28B). The intensity of TRPV1 expression was increased following WIN-55 treament (Figure 5.28B)

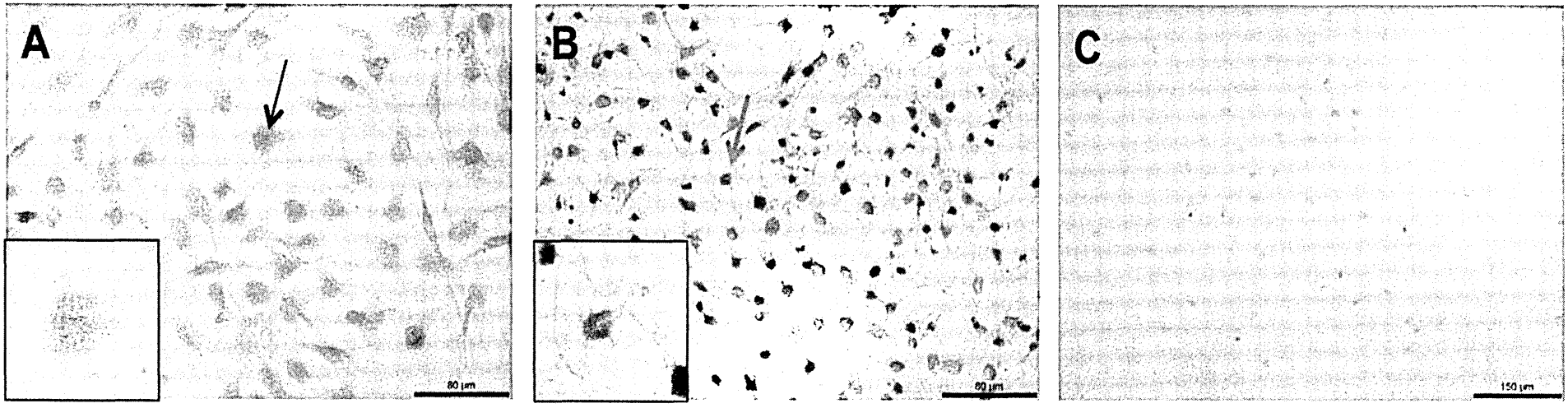

Figure 5.24 CB1 localisation in OA chondrocytes in HC21(4) monolayer culture. Immunopositivity is determined by the presence of brown staining. **(A)** DMSO control. **(B)** WIN-55 treatment for 48 hours. **(C)** IgG control (10 µg). Images show positivity is localised to the cytoplasm in DMSO control cells (black arrow). Following WIN-55 treatment positivity is localised to the nucleus (red arrow). Cells nuclei are counter stained with haematoxylin to allow for identification of negative cells.

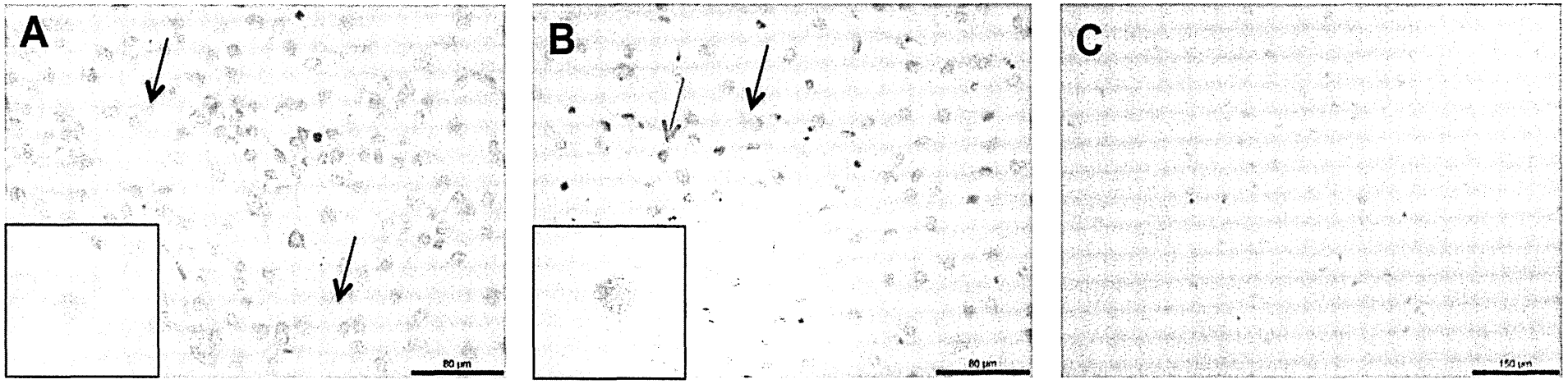

Figure 5.25 CB2 localisation in OA chondrocytes in HC21(4) monolayer culture. Immunopositivity is determined by the presence of brown staining. **(A)** DMSO control, **(B)** WIN-55 treatment for 48 hours, **(C)** IgG control (10 µg). Images show positivity is localised to the cytoplasm in DMSO control cells (black arrow). Following WIN-55 treatment positivity is localised to both the cytoplasm (black arrow) and nucleus (red arrow). Cells nuclei are counter stained with haematoxylin to allow for identification of negative cells.

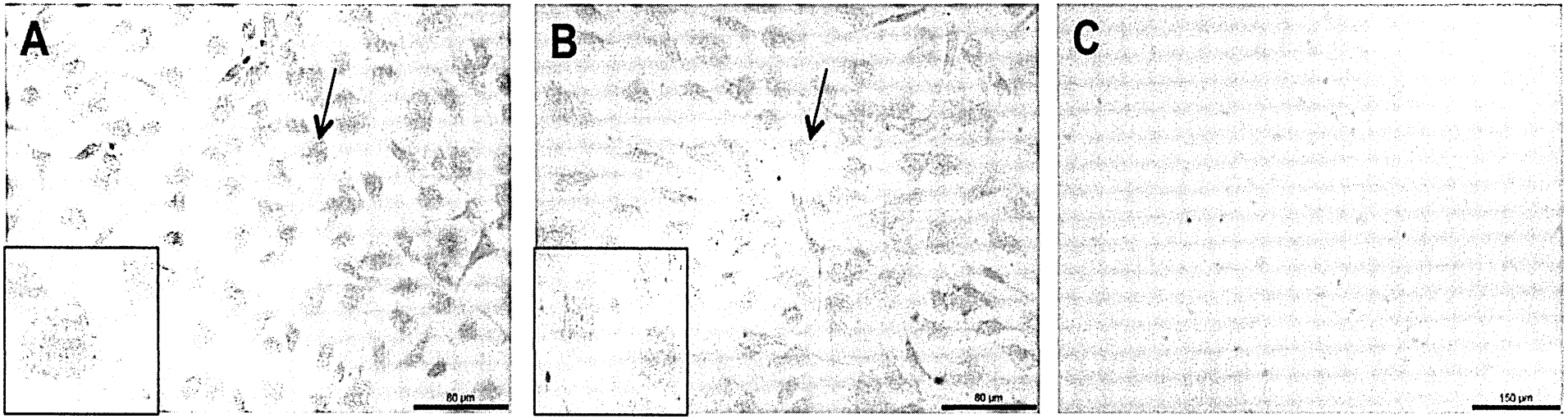

Figure 5.26 GPR55 localisation in OA chondrocytes in HC21(4) monolayer culture. Immunopositivity is determined by the presence of brown staining. **(A)** DMSO control, **(B)** WIN-55 treatment for 48 hours, **(C)** IgG control (10 µg). Images show positivity is localised to the cytoplasm in both DMSO control and WIN-55 treated cells (black arrow). Cells nuclei are counter stained with haematoxylin to allow for identification of negative cells.

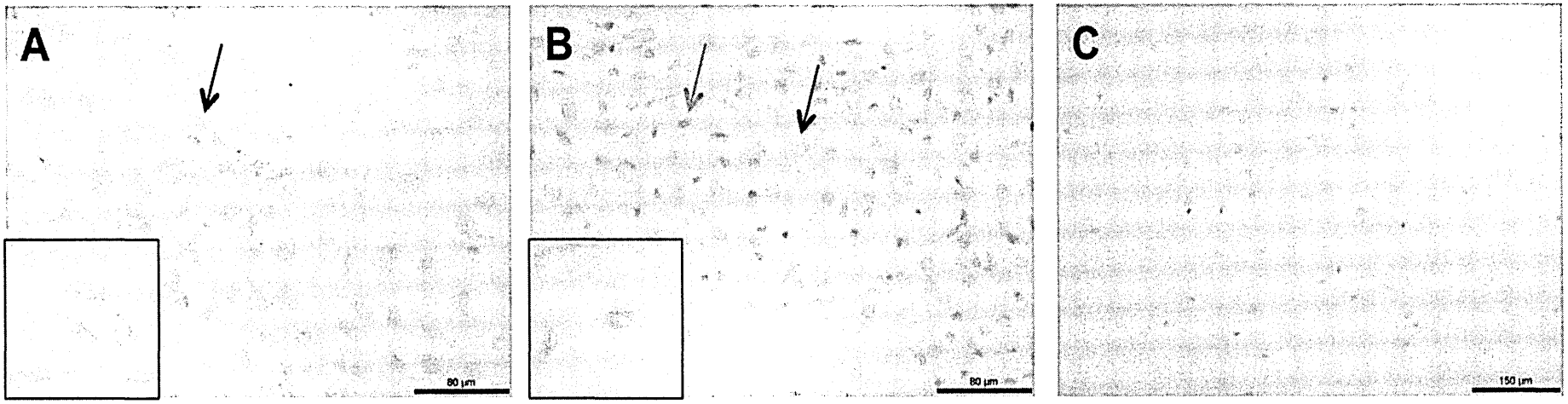

Figure 5.27 GPR18 localisation in OA chondrocytes in HC21(4) monolayer culture. Immunopositivity is determined by the presence of brown staining. **(A)** DMSO control, **(B)** WIN-55 treatment for 48 hours, **(C)** IgG control (10 µg). Images show positivity is localised to the cytoplasm in both DMSO control (black arrow). Following WIN-55 treatment positivity is localised to both the cytoplasm (black arrow) and nucleus (red arrow). Cells nuclei are counter stained with haematoxylin to allow for identification of negative cells.

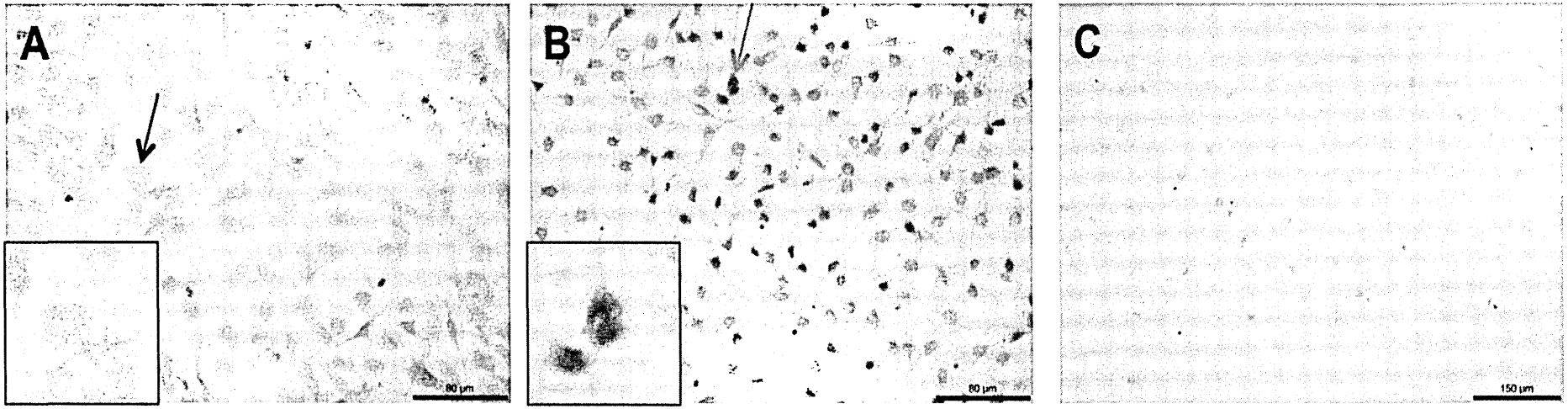

Figure 5.28 TRPV1 localisation in OA chondrocytes in HC21(4) monolayer culture. Immunopositivity is determined by the presence of brown staining. **(A)** DMSO control, **(B)** WIN-55 treatment for 48 hours, **(C)** 2° antibody alone. Images show positivity is localised to the cytoplasm in both DMSO control (black arrow). Following WIN-55 treatment positivity is localised to the nucleus (red arrow). Cells nuclei are counter stained with haematoxylin to allow for identification of negative cells.

5.5.2.6 PPARα Expression

PPARα expression was localised to both the cytoplasm and nucleus in the chondrocytes of the DMSO control (Figure 5.29A). Following WIN-55 treatment, PPARα expression was localised primarily to nuclei with cytoplasmic positivity also observed (Figure 5.29B). The intensity of PPARα expression was increased following WIN-55 treatment (Figure 5.29B)

5.5.2.7 PPARδ Expression

PPARδ expression was localised to primarily to the cytoplasm in the chondrocytes of the DMSO control with nuclei staining also observed (Figure 5.30A). Following WIN-55 treatment PPARδ expression was localised to the nuclei (Figure 5.30B). The intensity of PPARδ expression was increased following WIN-55 treatment (Figure 5.30B)

5.5.2.8 PPARγ Expression

PPARγ expression was localised to the cytoplasm of the chondrocytes in the DMSO control (Figure 5.31A). Following WIN-55 treatment for 48 hours PPARγ expression was still localised to cytoplasm, however a small number of cells also displayed nuclei staining (Figure 5.31B).

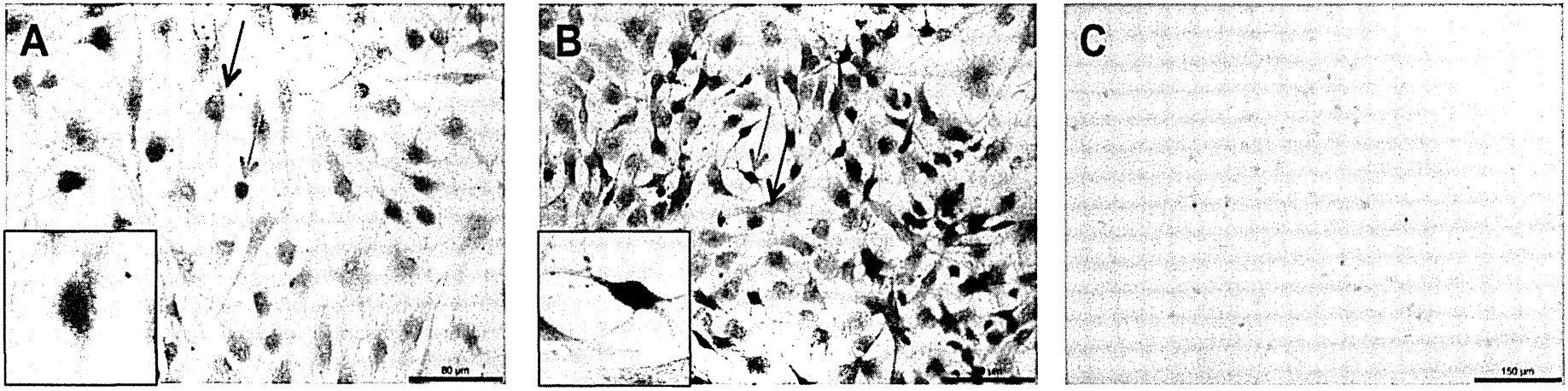

Figure 5.29 PPARα localisation in OA chondrocytes in HC21(4) monolayer culture. Immunopositivity is determined by the presence of brown staining. **(A)** DMSO control. **(B)** WIN-55 treatment for 48 hours **(C)** IgG control (10 µg). Images show positivity is localised to both the cytoplasm (black arrow) and nuclei (red arrow) of DMSO control cells. Following WIN-55 treatment positivity is localised primarily to the nucleus (red arrow) with cytoplasmic positivity also observed (black arrow). Cells nuclei are counter stained with haematoxylin to allow for identification of negative cells.

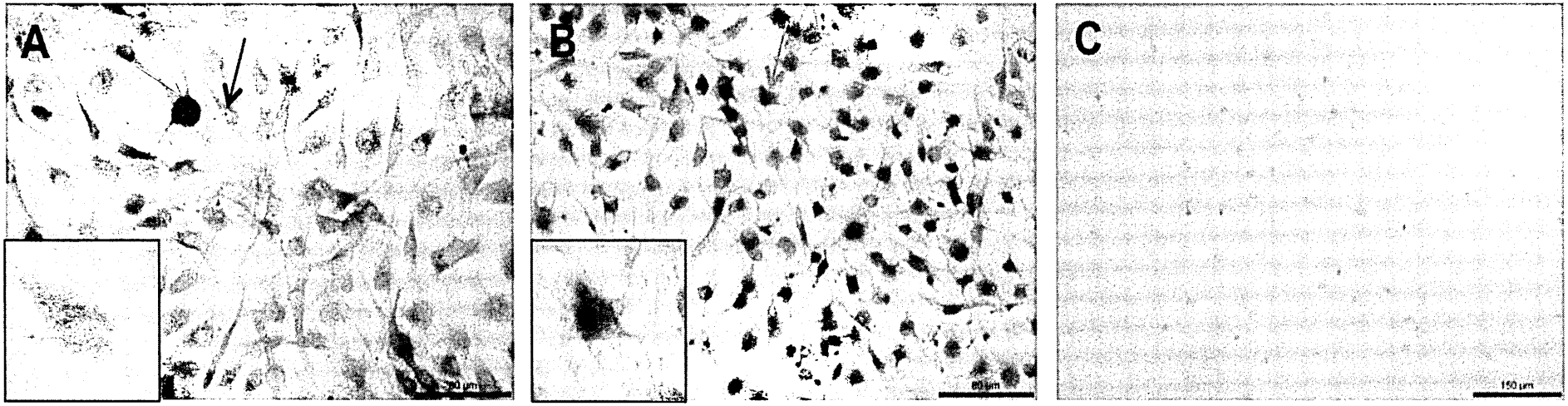

Figure 5.30 PPARδ localisation in OA chondrocytes in HC21(4) monolayer culture. Immunopositivity is determined by the presence of brown staining. **(A)** DMSO control. **(B)** WIN-55 treatment for 48 hours **(C)** IgG control (10 µg). Images show positivity is localised to both the cytoplasm (black arrow) and nucleus (red arrow) of DMSO control cells. Following WIN-55 treatment positivity is localised to the nucleus (red arrow). Cells nuclei are counter stained with haematoxylin to allow for identification of negative cells.

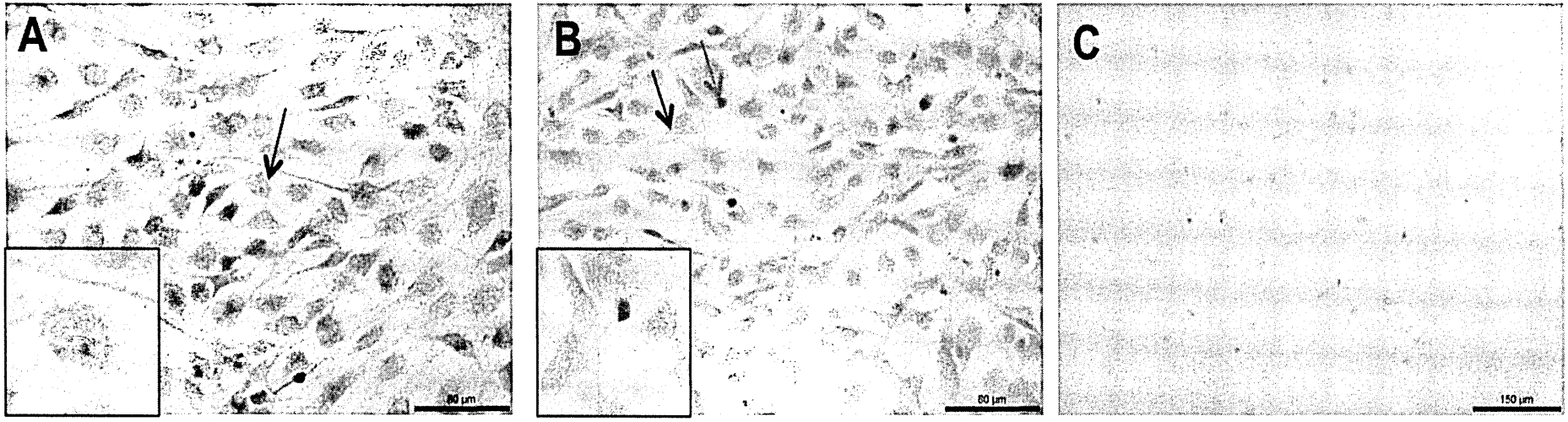

Figure 5.31 PPARγ localisation in OA chondrocytes in HC21(4) monolayer culture. Immunopositivity is determined by the presence of brown staining. **(A)** DMSO control, **(B)** WIN-55 treatment for 48 hours, **(C)** IgG control (10 µg). Images show positivity is localised to the cytoplasm (black arrow) of DMSO control cells. Following WIN-55 treatment positivity is still localised to the cytoplasm (black arrow), with a small number of cell also showing nuclei positive staining (red arrow). Cells nuclei are counter stained with haematoxylin to allow for identification of negative cells.

5.5.3 The Effects of WIN-55 on PPAR mRNA expression

5.5.3.1 PPARα

IL-1β stimulation for 48 hours had no significant effect on PPARα mRNA expression (Figure 5.32A). WIN-55 in combination with IL-1β for 48 hours significantly increased PPARα mRNA compared to DMSO control and IL-1β treatment alone ($p<0.001$) (Figure 5.32A). WIN-55 treatment alone for 48 hours significantly increased PPARα mRNA expression compared to DMSO control ($p<0.001$) (Figure 5.32A).

5.5.3.2 PPARδ

IL-1β stimulation for 48 hours had no significant effect on PPARδ mRNA expression (Figure 5.32B). WIN-55 in combination with IL-1β for 48 hours significantly increased PPARδ mRNA compared to DMSO control and IL-1β treatment alone ($p<0.001$) (Figure 5.32B). WIN-55 treatment alone for 48 hours significantly increased PPARδ mRNA expression compared to DMSO control ($p<0.001$) (Figure 5.32B).

5.5.3.3 PPARγ

IL-1β stimulation for 48 hours had no significant effect on PPARγ mRNA expression (Figure 5.32C). WIN-55 treatment both alone and in combination with IL-1β for 48 hours had no significant effect on PPARγ mRNA expression (Figure 5.32C).

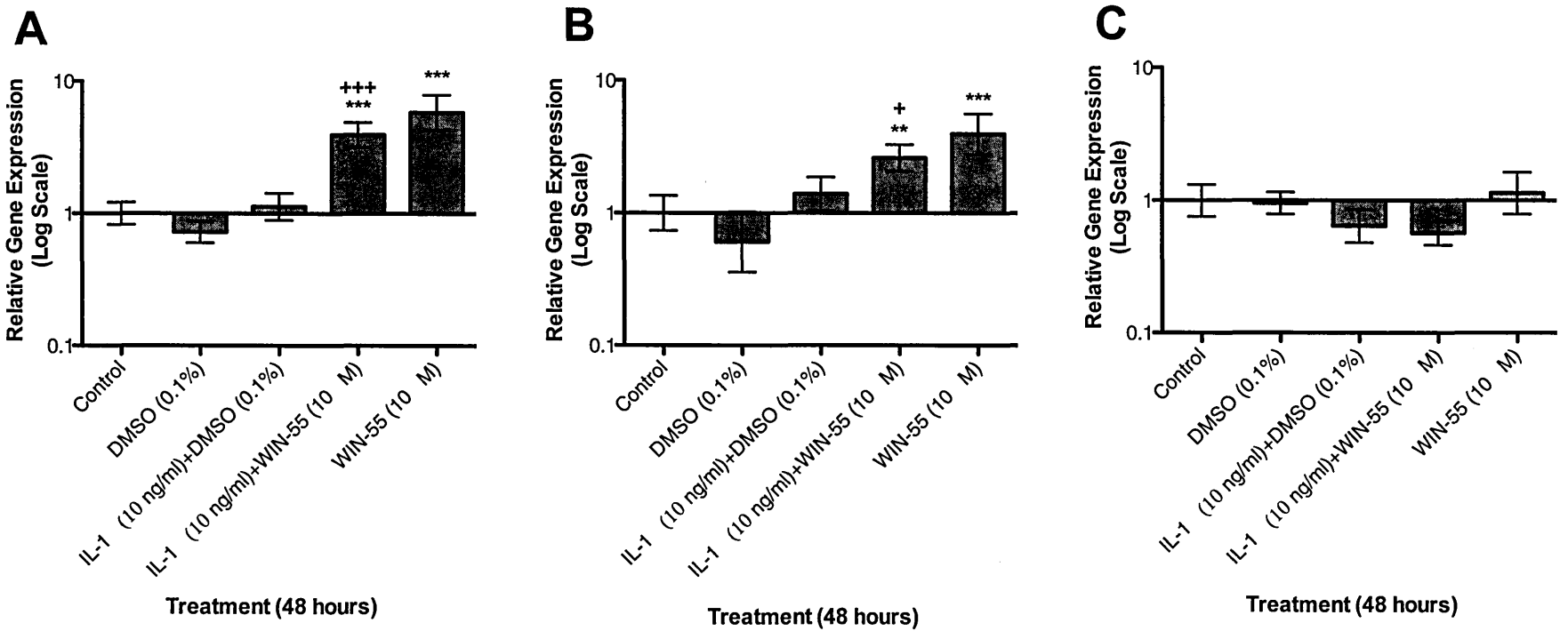

Figure 5.32 The effects of WIN-55 on PPAR mRNA expression. (A) PPARα mRNA expression. **(B)** PPARδ mRNA expression. **(C)** PPARγ mRNA expression. IL-1β stimulation had no significant effect of PPARα, δ or γ mRNA expression. Following WIN-55 treatment both alone and in combination with IL-1β PPARα and δ mRNA expression was significantly increased. WIN-55 both alone and in combination with IL-1β had no significant effect on PPARγ mRNA expression. Data represents mean fold change of mRNA expression normalised to internal reference gene and untreated control ± SEM. n=15 obtained for 5 patient samples. **p<0.01, ***p<0.001 compared to DMSO control and +p<0.05, ++p<0.001 compared to IL-1β treatment alone. Full patient details can be seen in Appendix 1.

5.5.4 Summary of Results

The summary of the findings presented in this chapter for immunohistochemical analysis of cannabinoid receptors in microscopically graded OA tissue and the effects of WIN-55 on cannabinoid receptor localisation in OA chondrocyte cultures and PPARα, δ and γ expression are shown in Tables 5.7 and 5.8.

Cannabinoid Receptor	Superficial Zone	Middle Zone	Deep Zone	Clusters	Osteocytes
CB1	–	–	–	–	–
CB2	–	–	–	–	–
GPR55	–	–	–	–	–
GPR18	–	–	↓	–	–
TRPV1	–	–	↓	–	–
PPARα	–	–	–	–	–
PPARδ	–	–	–	–	–
PPARγ	–	–	–	–	↓

Table 5.7 Summary of cannabinoid receptor expression in human OA cartilage in different zones of cartilage. – Indicates no change in receptor expression associated with grade of degeneration and ↓ indicates decrease in receptor with increasing grade of degeneration.

Cannabinoid Receptor	Cellular localization in DMSO control	Cellular localization following WIN-55 treatment	mRNA expression
CB1	Cytoplasm	Nucleus	Not investigated
CB2	Cytoplasm	Cytoplasm and nucleus	Not investigated
GPR55	Cytoplasm	Cytoplasm	Not investigated
GPR18	Cytoplasm	Cytoplasm and nucleus	Not investigated
TRPV1	Cytoplasm	Nucleus	Not investigated
PPARα	Cytoplasm and nucleus	Cytoplasm and predominantly nucleus	↑
PPARδ	Cytoplasm and nucleus	Predominantly nucleus	↑
PPARγ	Cytoplasm and nucleus	Cytoplasm and nucleus	–

Table 5.8 Summary of cannabinoid receptor localisation in OA chondrocyte cultures and the mRNA expression of PPARs following 10μM WIN-55 stimulation for 48 hours. – Indicates no change in mRNA expression compared to DMSO control and ↑ indicates a significant increase in mRNA expression compared to DMSO control.

5.6 Discussion

This work aimed to determine the expression of putative cannabinoid receptors in human articular chondrocytes and to identify if the expression of cannabinoid receptors within OA cartilage was grade and zone specific. In addition the effects of WIN-55 on the expression and localisation of cannabinoid receptors in OA chondrocytes cultured in monolayer was investigated. Finally, the effects of WIN-55 and IL-1β on PPARα, δ and γ mRNA expression in OA chondrocytes was also investigated.

Cells of the joint including chondrocytes, synovial fibroblasts, osteoblasts, osteoclasts and osteocytes express cannabinoid receptors including, CB1, CB2, GPR55, TRPV1 and PPARα δ and γ (Mbvundula *et al,* 2006; Richardson *et al,* 2008; Andersson *et al,* 2011; Whyte *et al,* 2009; Gavenis *et al,* 2009; Bordji *et al,* 2000; Shao *et al,* 2005; Fahmi *et al,* 2001; Fahmi *et al,* 2002; Afif *et al,* 2007; Moulin *et al,* 2005; Boyault *et al,* 2001; Selvi *et al,* 2008; Clockaerts *et al,* 2011a; Idris *et al,* 2005; Idris *et al,* 2009). Furthermore, activation and signalling mediated by cannabinoid receptors and cannabinoid agonists display chondroprotective and anti-arthritic activities both *in vivo* and *in vitro* (Mbvundula *et al,* 2006; Fahmi *et al,* 2001; Fahmi *et al,* 2002; Boyault *et al,* 2001; Zurier *et al,* 1998; Malfait *et al,* 2000; Sumariwalla *et al,* 2004; Mbvundula *et al,* 2005; Clockaerts *et al,* 2011; Bianchi *et al,* 2005; Kobayashi *et al,* 2005; Boileau *et al,* 2007; Francois *et al,* 2006; Lee *et al,* 2007; Poleni *et al,* 2010). Although cannabinoid receptors are expressed by joint cells involved in the pathogenesis of OA their expression levels in different cartilage zones and bone during degradation is poorly defined.

5.6.1 CB1 and CB2

In the present study the expression of both classical cannabinoid receptors CB1 and CB2 in human OA cartilage was observed, although their percentage immunopositivity did not change with grade of degeneration within chondrocytes in the different zones of cartilage or osteocytes. Interestingly, both CB1 and CB2 expression was significantly higher in chondrocytes within the different zones of cartilage compared to osteocytes and immunopositivity of CB2 was less than that of CB1 in both chondrocytes and osteocytes. CB1 and CB2 receptors have previously been shown to be expressed by bovine articular chondrocytes (Mbvundula *et al,* 2006). In addition CB1 and CB2 are expressed

at similar levels both at the protein and RNA level in synovia of patients with OA and RA and their expression is thought to play a role in the pathology of joint disease (Richardson *et al,* 2008). Other studies have shown that both CB1 and CB2 are expressed at the protein level in human chondrocytes and fibroblast-like synoviocytes, although the role of these receptors in modulating chondrocyte metabolism in unknown (Andersson *et al,* 2011; Selvi *et al,* 2008). CB2 expression is increased in chronic pain models associated with peripheral nerve injury; principally in the spinal cord suggesting that it may be a therapeutic target (Zhang *et al,* 2003; Wotherspoon *et al,* 2005; Yiangou *et al,* 2006). However, here it was shown that the expression of CB2 did not change in OA chondrocytes or osteocytes with grade of degeneration.

Immunohistochemical staining demonstrated that CB1 and CB2 expression was localised to the nucleus and perinucleus of chondrocytes. Although, CB1 and CB2 are cell membrane receptors, they have been shown to redistribute and traffick to different cellular components following ligand binding (Abood 2005). Endocannabinoids including AEA and 2-AG are found in the synovial fluid of human OA but not normal patients (Richardson *et al,* 2008). Therefore, it is possible that endogenous cannabinoid ligands present in the synovial fluid may activate cannabinoid receptors expressed by chondrocytes in OA articular cartilage, subsequently inducing their internalisation and redistribution under pathological condtions. However, whether the classical cannabinoid receptors are expressed in the nucleus or perinuclear at basal levels, under non-ligand activated conditions in chondrocytes remains to be determined.

Bone remodelling occurs in OA at sites of bone damage and thickening of the subchondral bone plate is a pathological feature of OA (Goldring and Goldring 2010). This study has shown that both the classical cannabinoid receptors CB1 and CB2 are expressed by OA osteocytes; however expression in osteocytes did not change with OA grade of degeneration. Studies have shown the importance of cannabinoid receptors and cannabinoid ligands in the regulation of bone metabolism. Both CB1 and CB2 are involved in bone metabolism, regulating bone mass and loss via the modulation of cell function (Idris and Ralston 2010). CB1 and CB2 receptors have previously been shown to be expressed by mouse and human osteoclasts, osteoblasts and osteocytes (Idris *et al,* 2005; Ofek *et al,* 2006; Rossi *et al,* 2009; Whyte *et al,* 2012). CB1

knockout mice have increased bone density but develop age related osteoporosis and both CB1 and CB2 knockout mice are protected against ovariectomy induced bone loss compared to wild type mice (Idris *et al,* 2005; Idris *et al,* 2009; Idris *et al,* 2008). Ofek *et* al (2006) demonstrated that CB2 deficient mice had increased bone turnover and mice developed age related osteoporosis. The CB1 selective antagonist AM251 and the CB2 selective antagonists AM630 and SR144528, when used in the nanomolar range, inhibited osteoclast formation (Idris *et al,* 2005; Idris *et al,* 2008). Osteoclast cultures from CB1 knockout mice were found to be resistant to the inhibitory effects of AM251 on osteoclast activity, suggesting that osteoclast inhibition is mediated by CB1 (Idris *et al,* 2005). Moreover CB1 and CB2 receptor agonists AEA, CP55940, HU308 and JWH133 stimulated osteoclast formation thus suggesting that cannabinoid receptor agonists may stimulate bone resorption thus enhancing bone loss (Idris *et al,* 2005; Idris *et al,* 2008). In contrast, CB2 antagonist AM630 used at higher concentrations in human osteoclast cultures stimulated osteoclast formation and CB2 agonist HU308 inhibited receptor activator of nuclear factor kappa-B ligand (RANKL) induced osteoclast formation (Ofek *et al,* 2006; Rossi *et al,* 2009). In a later study, human osteoclast differentiation was found to be associated with a decrease in CB2 receptor and endogenous cannabinoid 2-AG and an increase in endogenous cannabinoid AEA (Whyte *et al,* 2012). Moreover 2-AG and AEA were found to stimulate bone resorption in human osteoclast cultures (Whyte *et al,* 2012). *In vivo,* CB2 specific inverse agonist Sch.036 prevented bone damage in a rat model of arthritis (Lunn *et al,* 2007). Such conflicting findings on CB1 and CB2 expression in bone suggest that the actions of cannabinoid agonists or antagonist at receptors expressed by bone cells may be species specific or produce different effects depending on the concentration used.

Previous studies have focused on expression and function of cannabinoid receptors and agonists in osteoclasts. However, studies have shown that osteocytes embedded within the bone matrix signal to osteoblasts and osteoclasts, regulating their activity in bone turnover and remodelling (Manolagas 2000; Sims and Gooi 2008). In the present study the percentage expression of cannabinoid receptors in osteocytes during cartilage degeneration has been determined. Here it was shown that there was a higher

percentage of CB1 positive osteocytes compared to CB2 positive osteocytes in OA bone. In contrast previous studies have shown that CB2 is expressed at higher levels in osteoclasts, osteoblasts and osteocytes compared to CB1 (Idris *et al,* 2005; Ofek *et al,* 2006; Rossi *et al,* 2009; Whyte *et al,* 2012). During OA osteoblasts anabolic activity associated with bone remodelling is increased compared to that of normal osteoblasts, thus contributing to increases in subchondral bone thickness and the formation of osteocytes (Hilal *et al,* 1998; Truong *et al,* 2006). CB1 knockout mice displayed decreased osteoblast differentiation resulting in a decrease in age-related bone loss (Idris *et al,* 2009). Together these finding suggest that targeting the cannabinoid system may be of therapeutic value in the treatment of bone diseases such as osteoporosis via the modulation of osteoclast and osteoblast activity. However, the relevance of CB1 and CB2 expression by bone cells during OA, where an increase in bone formation and remodelling is a pathological feature, has yet to be determined.

In monolayer culture of DMSO control cells, immunopositivity of CB1 was observed in the cytoplasm. Upon stimulation with WIN-55 for 48 hours, CB1 expression was predominately localised to the nucleus. Upon binding of agonists, GPCRs are rapidly desensitised and internalised (Abood 2005). Trafficking of GPCRs involves phosphorylation of the receptor, the binding of the cytoplasmic scaffold protein β-arrestin with the receptor which results in the recruitment of clathrin-coated pits, followed by endocytosis (Shenoy and Lefkowitz 2003). The receptors are finally recycled and re-distributed to the membrane or are degraded by lysosomal enzymes (Shenoy and Lefkowitz 2003). WIN-55 activates CB1 and CB2 with Kis of 1.89-123 nM and 0.28-16.2 nM respectively (Pertwee *et al,* 2010). Hsiesh *et al* (1999) showed that following WIN-55 stimulation CB1 was internalised as indicated by punctated immunopositivity inside the pituitary cell line AtT20 stably expressing CB1 and internalisation was blocked using CB1 antagonist SR141716A. Internalisation of CB1 by WIN-55 was found to be rapid, occurring 5 minutes after stimulation, however after 60 minutes WIN-55 stimulated intracellular staining was decreased suggesting degradation of CB1 (Hsieh *et al,* 1999). The removal of WIN-55 following short periods of stimulation resulted in the re-distribution of CB1 to the cell membrane; however following long periods of stimulation the synthesis of new CB1 protein is required as shown by a decrease in intracellular

staining (Hsieh *et al,* 1999). Interestingly, in the present study, nuclear localisation of CB1 following WIN-55 treatment was observed following 48 hours stimulation, therefore the mechanism of this observed cellular re-distribution remains to be determined.

WIN-55 also binds to and activates CB2 receptors (Howlett *et al,* 2002) Interestingly CB2 agonist CP55-940 but not WIN-55 induced CB2 internalisation in HEK293 cells stably expressing CB2 (Atwood *et al,* 2012). Moreover WIN-55 antagonised CP55-940 induced CB2 internalisation (Atwood *et al,* 2012). However, WIN-55 was shown to activate CB2 as shown by ERK1/ERK2 phosphorylation and β-arrestin$_2$ membrane recruitment, suggesting that WIN-55 displays functional selectivity at CB2 (Atwood *et al,* 2012). Data presented in this study showed that following WIN-55 stimulation of OA chondrocytes for 48 hours CB2 expression appeared to be localised to both the cytoplasm with nuclear staining also observed, although cellular re-distribution of CB2 was not as predominant as that of CB1. Further investigation is required in order to fully elucidate the mechanism via which WIN-55 induces CB1 and CB2 cellular redistribution and localisation in human OA chondrocytes.

5.6.2 GPR55

GPR55 has been identified as a possible cannabinoid receptor as it is activated by a number of cannabinoid receptor agonists (Ryberg *et al,* 2007). In the present study GPR55 was ubiquitously expressed by both chondrocytes and osteocytes. In addition both osteoclasts and osteoblasts expressed GRP55, however this was not quantified. GPR55 is expressed by both normal and OA chondrocytes (Andersson *et al,* 2011), however, there is little knowledge regarding its role in chondrocyte metabolism. Interestingly in this present study there was no significant change in GPR55 expression with grade of degeneration in the different zones of the cartilage or the bone. Similarly, as shown with CB1 and CB2 expression, GPR55 immunopositivity in osteocytes was significantly less than expression in the chondrocytes of the middle and deep zone and clusters of the cartilage.

In addition to CB1 and CB2, GPR55 is thought to play a role in bone metabolism (Idris and Ralston 2010). GPR55 is expressed in human and mouse osteoclasts and osteoblasts (Whyte *et al,* 2009). Adult GPR55 deficient mice

have an osteoclastogenesis defect resulting in increased peak bone mass, without changes in osteoblast number (Whyte *et al,* 2009). Moreover cannabinoid agonist O-1602 and non-cannabinoid ligand LPI were found to inhibit osteoclast formation in an *in vitro* mouse model; however these ligands also stimulated osteoclast function (Whyte *et al,* 2009). Interestingly GPR55 antagonist phytocannabinoid CBD reduced bone resorption in a mouse model (Whyte *et al,* 2009).

GPR55 has been identified as a possible cannabinoid receptor as it is activated by a number of cannabinoid receptor agonists (Ryberg *et al,* 2007). Moreover, GPR55 has been shown to internalise following incubation with CB1 antagonist AM251 and SR141716A and non-cannabinoid ligand LPI (Kapur *et al,* 2009). In the present study, WIN-55 stimulation had no effect on GPR55 localisation or redistribution in OA chondrocytes in monolayer culture compared to the DMSO control. Findings presented here are in agreement with other studies which have shown WIN-55 does not display activities at GPR55 (Kapur *et al,* 2009; Pertwee 2007).

5.6.3 GPR18

Recently GPR18 has been shown to be activated by endogenous cannabinoid AEA, NAGly a metabolite of AEA and phytocannabinoid THC in HEC-1B cells (McHugh *et al,* 2012; McHugh *et al,* 2010). GPR18 is primarily expressed in testes and spleen and in addition is expressed in other tissues and cells involved in endocrine and immune functions including, peripheral blood leukocytes, small intestine and the thymus (Gantz *et al,* 1997). In the present study it is shown that GPR18 was expressed by chondrocytes in OA cartilage and osteocytes and in the deep zone of the cartilage GPR18 expression was decreased with grade of degeneration. In addition there was a significant increase in the percentage of chondrocytes expressing GPR18 in the middle and deep zone compared to the superficial zone of the cartilage. These findings indicate GPR18 may play a role in the pathogenesis of OA and may have different functions in the middle and deep zone of the cartilage, as chondrocytes in different zones are known to express different molecules and display distinct functions (Goldring and Marcu 2009).

Immunocytochemical studies here have shown that in DMSO control chondrocytes, GPR18 expression is localised to the cytoplasm of the cells and following WIN-55 treatment a small proportion of cells displayed nuclear staining. Interestingly cannabinoid WIN-55 had no effect on GRP18 signalling in previous studies (McHugh *et al,* 2012). Data presented here is the first reported finding of GPR18 in cartilage and bone tissue and its role in chondrocyte and bone metabolism under both physiological and pathological conditions remains to be investigated.

5.6.4 TRPV1

TRPV1 acts as an endogenous cannabinoid receptor for AEA and has also been shown to bind phytocannabinoids including CBD (Bisogno *et al,* 2001; Smart and Jerman 2000). TRPV1 was mainly expressed by nociceptive neurons and is activated by noxious heat and capsaicin (Caterina *et al,* 1997; Benham *et al,* 2003). Interestingly TRPV1 expression has been associated with arthritis pain in animal models. TRPV1 was increased in a rat OA model compared to control animals (Fernihough *et al,* 2005). Furthermore, TRPV1 knockout mice have reduced thermal hyperalgesic sensitivity in an adjuvant-induced arthritis model (Keeble *et al,* 2005). TRPV1 is expressed in human OA chondrocytes at the mRNA level and human OA and RA synovial fibroblasts at both the mRNA and protein level (Gavenis *et al,* 2009; Engler *et al,* 2007). TRPV1 is associated with pain in OA as shown by animal models (Keeble *et al,* 2005; Fernihough *et al,* 2005). It may be postulated that an increase in TRPV1 expression would be associated with OA disease progression particularly in the deep zone of the cartilage since the osteochondral junction is thought to be the source of pain in OA and angiogenesis has been associated with NGF expression in human OA patients (Walsh *et al,* 2010). Interestingly, in the present study it was shown that TRPV1 expression was significantly decreased in the deep zone of the cartilage in severe degeneration when compared to low degeneration OA patient samples. However, a decrease in TRPV1 expression has been associated with a differentiated phenotype in human OA chondrocytes cultures (Gavenis *et al,* 2009), suggesting that a decrease in TRPV1 expression observed in OA cartilage here may be due to more differentiated chondrocytes in the deep zone in severe degeneration in OA (Goldring 2012).

TRPV1 expression has also been associated with bone metabolism. TRPV1 is expressed in human osteoclast cultures and human bone tissue and TRPV1 agonists resiniferatoxin (RTX) and capsaicin increased osteoclast number and intracellular Ca^{2+} in human osteoclast cultures (Rossi *et al,* 2009). Interestingly TRPV1 was found to be co-localised with CB1 and CB2 receptors, suggesting cross talk occurs between these receptors in osteoclasts (Rossi *et al,* 2009). These findings suggest that TRPV1 may be involved in osteoclast activity and bone resorption. The role of TRPV1 in cartilage breakdown and bone resorption in OA has yet to be determined.

In the present study, in monolayer culture, TRPV1 was expressed in the cytoplasm of the chondrocytes. Following WIN-55 treatment, expression was predominantly localised to the nuclei compared to the DMSO control, suggesting that WIN-55 induced the cellular redistribution of TRPV1 expression in human OA chondrocytes. In primary rat trigeminal ganglion cultures, WIN-55 treatment lead to the dephosphorylation of TRPV1 at Thr144 and Thr370 resulting in the desensitization of TRPV1 (Jeske *et al,* 2006), suggesting that WIN-55 induces analgesic activities via TRPV1 activation and desensitization (Jeske *et al,* 2006). Moreover, desensitization of TRPV1 occurs via the internalisation of the receptor by endocytosis and lysosomal degradation, independent of the clathrin pathway (Sanz-Salvador *et al,* 2012). Therefore internalisation of TRPV1 by WIN-55 treatment in the present study, as shown by intracellular immunopositivity, suggests that WIN-55 may reduce the levels of TRPV1 expressed on the membrane of cells resulting in reduction of pain signalling.

5.6.5 PPARα, δ and γ

Other candidates for cannabinoid receptors include the PPARs. Targeting PPARs using specific agonists and cannabinoid agonists for the treatment of OA and RA has been reported previously (O'Sullivan and Kendall 2010; Fahmi *et al,* 2001; Fahmi *et al,* 2002; Johnson *et al,* 2007; Clockaerts *et al,* 2011; Fahmi *et al,* 2011; Giaginis *et al,* 2009). Endogenous cannabinoid ligands AEA, OEA, PEA, noladin ether and virodhamine and synthetic cannabinoid WIN-55 display PPARα binding and promote transcriptional activity (Sun *et al,* 2006; Lo Verme *et al,* 2005; Fu *et al,* 2003). Little is known regarding the effects of cannabinoids on PPARδ, however, OEO increased transcriptional activity of

PPARδ (Fu *et al,* 2003). Cannabinoid AJA, a THC analogue, has been shown to bind to the ligand-binding domain of human PPARγ (Ambrosio *et al,* 2007). AJA also reduced joint damage in an animal arthritis model (Zurier *et al,* 1998) and has been shown to have anti-inflammatory effects by inhibiting the promoter activity of IL-8 (Liu *et al,* 2003). These effects may be PPARγ mediated, suggesting a role for the involvement of these receptors in cannabinoid-mediated chondroprotection.

In the present study, there was a trend towards increased expression of PPARγ in chondrocytes in the superficial zone of cartilage and immunopositivity was predominantly localised to the nucleus. In agreement with the current study, PPARγ was previously shown to be predominantly expressed in the superficial zone of human OA cartilage and PPARγ expression has been shown to be nuclear in rat cartilage (Bordji *et al,* 2000; Afif *et al,* 2007). However increased staining for PPARγ observed in the superficial zone of the cartilage may be due to increased cellularity compared to the middle and deep zone. Interestingly, PPARγ expression has been shown to be down regulated in human OA cartilage compared to normal cartilage, suggesting that PPARγ is involved in cartilage ECM metabolism (Afif *et al,* 2007). In addition, *In vivo,* PPARγ expression is decreased during the progression of OA (Nebbaki *et al,* 2013). In contrast we have shown that there was no significant difference in PPARγ expression in OA cartilage with grade of degeneration.

In animal models of arthritis the PPARγ agonist piogitazone reduced the development and severity of cartilage lesions (Kobayashi *et al,* 2005; Boileau *et al,* 2007) and the importance of PPARγ in cartilage development and homeostasis has recently been investigated (Vasheghani *et al,* 2013; Monemdjou *et al,* 2012). In cartilage specific PPARγ knockout in newborn mice, ablation of PPARγ resulted in reduced skeletal growth, body weight and length of long bones compared to control mice (Monemdjou *et al,* 2012). In addition, mice demonstrated growth plate defects, delayed ossification and reduced cellularity of the cartilage, loss of columnar organisation, altered chondrocyte shape and a shorter hypertrophic zone compared to control mice (Monemdjou *et al,* 2012). Reduced chondrocyte proliferation, differentiation, hypertrophy and vascular invasion were also demonstrated by a decrease in immunohistochemical markers: Sox-9, BrdU, p27 and collagen type X

compared to control mice (Monemdjou *et al,* 2012) Moreover, chondrocytes and cartilage explants isolated from PPARγ knockout newborn mice showed a decrease in expression of aggrecan and collagen type II and increased expression of MMP-13 compared to control mice (Monemdjou *et al,* 2012). In another study by the same group, adult PPARγ cartilage-specific knockout mice exhibited a spontaneous OA phenotype (Vasheghani *et al,* 2013). PPARγ cartilage-specific knockout mice displayed histological features of OA including, cartilage degradation, increased proteoglycan loss, hypocellularity, calcified cartilage, fibrillation, synovial inflammation and fibrosis compared with age-matched controls (Vasheghani *et al,* 2013). Immunohistochemical analysis of PPARγ deficient cartilage demonstrated an increase in collagen and aggrecan neoepitopes C1-2C and VDIPEN respectively indicative of ECM breakdown, in addition an increase in MMP-13 and HIF2α expression was observed (Vasheghani *et al,* 2013). The mRNA for catabolic markers associated with OA was also increased in PPARγ knockdown cartilage-specific explants including, MMP-13, COX-2, NO and ADAMTS-5 but interestingly not ADAMTS-4 (Vasheghani *et al,* 2013). Together these findings suggest that PPARγ plays an important role in cartilage metabolism.

All three subtypes of PPARs have been identified in bone cells (Giaginis *et al,* 2007). Although in the present study there was no significant change in PPARγ expression in OA cartilage associated with grade of degeneration, it was shown that there was a significant decrease in PPARγ expression in osteocytes with increasing grade of degeneration. Cartilage specific PPARγ deficient mice displayed bone defects including reduced length of long bones, bone density and trabecular bone thickness (Monemdjou *et al,* 2012), suggesting that PPARγ is involved in bone metabolism. However, PPARγ agonists rosiglitazone and pioglitazone reduced bone erosions and inflammatory bone loss in a collagen induced arthritis model (Koufany *et al,* 2008) and PPARγ signalling pathway genes are up-regulated during the osteoblast mineralisation process (Staines *et al,* 2013). Although reduced expression of PPARγ is thought to play a role in the pathogenesis of OA (Afif *et al,* 2007; Nebbaki *et al,* 2013; Vasheghani *et al,* 2013), its role in bone metabolism during OA remains to be defined.

The present study has shown that both PPARα and δ are ubiquitously expressed by human OA chondrocytes and osteocytes. There was no

significant difference in PPARα or δ expression observed between different microscopic grades of OA cartilage. In agreement with our study, Afif *et al* (2007) showed that PPARα or δ was expressed by human articular chondrocytes and no differences in expression were observed in different grades of OA. *In vivo* studies have also shown that PPARα and δ expression levels did not changes during the progression of OA in animal models (Nebbaki *et al,* 2013).

This study showed that human OA osteocytes expressed both PPARα and δ, however no changes in PPAR α or δ positivity in osteocytes were observed with increasing degradation. PPARα deficient mice display no apparent bone abnormalities, suggesting PPARα is not involved in bone metabolism (Wu *et al,* 2000). However, PPARα agonists are known to increase bone density and stimulate osteoblast proliferation and differentiation (Still *et al,* 2008; Syversen *et al,* 2009). Furthermore, *in vivo* PPARδ agonists up-regulate osteoblast differentiation and induced periosteal bone formation (Still *et al,* 2008). However the role of PPARs in regulating bone metabolism in OA has yet to be determined.

In the present study, IL-1β had no effect on mRNA expression of PPARα, δ or γ in human OA chondrocytes. In agreement with these findings, IL-1β stimulation had no effect on PPARα in human OA cartilage explants, rat chondrocytes or rat synovial fibroblasts (Bordji *et al,* 2000;Moulin *et al,* 2005;Clockaerts *et al,* 2011). In addition IL-1β had no effect on PPARδ expression in rat synovial fibroblasts (Moulin *et al,* 2005). However, in contrast, IL-1β decreased PPARγ expression in OA chondrocytes and rat synovial fibroblasts (Afif *et al,* 2007;Moulin *et al,* 2005;Boyault *et al,* 2001). In OA chondrocytes, reporter gene assays showed that down-regulation of PPARγ by IL-1β was at the transcription level and activation of c-Jun, p38 and NFκB signalling pathways by IL-1β induced a decrease in PPARγ expression (Afif *et al,* 2007). In contrast, IL-1β induced PPARγ expression in human articular chondrocytes (Shan *et al,* 2004).

PPARγ is known to play a role in modulating catabolic factors in OA and targeting this receptor with specific ligands has been investigated. 15d-PGJ_2 a ligand for PPARγ decreased or abolished IL-1β induced mRNA expression of COX-2 and iNOS and release of prostaglandins and NO in human chondrocytes (Boyault *et al,* 2001). Furthermore, WIN-55 is known to activate

PPARγ (O'Sullivan and Kendall 2010; O'Sullivan 2007). Interestingly, in the present study, WIN-55 had no effect of PPARγ mRNA expression or cellular localisation in human OA chondrocytes. Immunocytochemical staining presented here showed that expression of PPARγ appeared to be both cytoplasmic and nuclear. Although PPARγ is a nuclear receptor, it is now evident that expression in not exclusively in the nucleus. Recent studies have shown that PPARγ shuttles between cellular compartments. Upon mitogen stimulation MEKs translocate to the nucleus, bind to PPARγ via interaction of the basic D domain of MEK and the AF2 domain of PPARγ and transport it to the cytoplasm of the cells (Umemoto and Fujiki 2012; Burgermeister *et al,* 2007). However, in this study PPARγ cytoplasmic staining was observed in unstimulated cells, suggesting that cytoplasmic PPARγ is also present without ligand activation, although cytoplasmic staining could also be due to endogenous ligands present at basal levels.

Activation of PPARα in human OA cartilage explants using a specific agonist Wy14643 resulted in a decrease in IL-1β induced expression of proinflammatory mediators MMP-1, -3 -13, NO and PGE_2 (Clockaerts *et al,* 2011). Moreover Wy14643 decreased IL-1β induced NFκB phosphorylation and translocation in OA chondrocytes (Clockaerts *et al,* 2011). These findings suggested that activation of PPARα may be chondroprotective and decrease both destructive and inflammatory factors in OA. In the present study an increase in nuclear PPARα positivity and mRNA expression was observed in OA chondrocytes treated with WIN-55. This finding was in agreement with Sun *et al* (2006) who showed that WIN-55 binds to and increases the transcriptional activity of PPARα. Similar to PPARγ, expression of PPARα is also predominantly localised to the cytoplasm of the chondrocytes. PPARs are nuclear receptors however studies have shown that the localisation of PPARα in chondrocytes is also cytoplasmic (Bordji *et al,* 2000). Furthermore, like PPARγ, PPARα also shuttles between the cytoplasm and nucleus following ligand activation (Umemoto and Fujiki 2012). The findings in the present study suggest that WIN-55 treatment retains PPARα in the nucleus. Consequently, since chondroprotective activities have been shown to be mediated by PPARα activation (Clockaerts *et al,* 2011), an increase in its mRNA expression and

nuclear localisation induced by WIN-55 may further reduce the expression of catabolic and inflammatory mediators in chondrocytes.

PPAR δ expression has previously been reported in rat and human cartilage, although there is little known about its role in chondrocyte metabolism (Shao *et al,* 2005; Afif *et al,* 2007). Interestingly, activation of PPARδ in rat synovial fibroblasts was shown to stimulate production of IL-1Ra, suggesting that PPARδ may have potential anti-arthritic properties (Moulin *et al,* 2005). The present study has shown that WIN-55 increases the mRNA expression of PPARδ in human OA chondrocytes. Moreover WIN-55 increased the intensity of PPARδ immunopositivity and an increase in nuclear staining was observed. However the effects of PPARδ activation on chondrocyte metabolism have yet to be determined.

5.6.6 Summary

The present study has demonstrated that cannabinoid receptors CB1, CB2, GPR55, GPR18, TRPV1 and PPARα, δ and γ are expressed by chondrocytes and osteocytes in OA cartilage and bone tissue respectively. Cannabinoid receptor expression in cartilage does not appear to be associated with grade of degeneration. Multiple studies have shown the potential of cannabinoid receptor activation to provide chondroprotective activities in arthritis. Maintenance of cannabinoid receptor expression shown in this study during OA suggests these cells would remain responsive. The present study showed that cannabinoid receptors exist in both the cartilage and bone; it is therefore possible that one or more of the cannabinoid receptors shown to be expressed here or indeed a yet unidentified cannabinoid receptor may mediate cannabinoid ligand actions. In this study, WIN-55 affected the cellular localisation and protein expression of CB1, CB2, GPR18, TRPV1 and PPARα and δ in OA chondrocyte cultures. It appeared to have affects on internalisation and trafficking of a number of cannabinoid receptors. Finally we have shown that WIN-55 induces the mRNA expression of PPARα and δ, therefore WIN-55 may have an additive effect on the PPAR receptor agonists, which have previously been shown to mediate chondrocyte protective activities. However, in order to further elucidate the effects of WIN-55 on cannabinoid receptor expression, cellular localisation and activation within OA chondrocytes further investigation is required.

6 Cannabinoid Receptor Agonists

6.1 Introduction

WIN-55 was shown to inhibit IL-1β induced MMP-3 and MMP-13 mRNA and protein expression and IL-1β induced phosphorylation of ERK1/ERK2, c-Jun and IκB in human OA chondrocytes (Chapter 2 and 3). In addition cannabinoid receptors CB1, CB2, GPR55, GPR18, TRPV1 and PPARα, δ and γ were shown to be expressed in OA cartilage (Chapter 4). Thus, the present study aimed to investigate if specific agonists for cannabinoid receptors could induce responses previously observed with WIN-55, in order to determine the receptors via which WIN-55 may mediated its effects.

6.1.1 CB1 and CB2 Receptor Agonists: ACEA and HU308

Endogenous cannabinoid AEA analogue, arachidonyl-2'-chloroethylamide (ACEA) is a selective agonist at the CB1 receptor which, similar to AEA undergoes enzymatic hydrolysis (Pertwee 2006). HU308 is a CB2 selective agonist and *in vivo,* is known to exhibit anti-inflammatory and peripheral analgesic properties (Hanus *et al,* 1999). In bovine chondrocytes, activation of CB1 and CB2 using synthetic agonist HU210, which is known to activate both of the classical receptors, was shown to inhibit IL-1α induced ECM degradation (Mbvundula *et al,* 2006). These findings suggest that activation of CB1 or CB2 may prevent ECM breakdown.

6.1.2 GPR55 Receptor Agonist: LPI

The natural ligand of GPR55 is thought to be LPI (Kapur *et al,* 2009). LPI induced the formation of β-arrestin-GPR55 complex and ERK1/ERK2 phosphorylation in GPR55-HEK293 (Kapur *et al,* 2009) and in contrast to CB1 and CB2 receptors, which involve Gi/o coupling, activation of GPR55, by LPI is thought to involve the coupling of Gα13 in GPR55-HEK293 cells (Henstridge *et al,* 2009). There is currently no information as to the effects of GPR55 activation on chondrocyte metabolism, although, the role of GPR55 has been investigated in inflammatory and neuropathic pain (Staton *et al,* 2008). These investigations demonstrated that in GPR55 knockout mice mechanical hyperalgesia was absent following intraplantar Freund's complete adjuvant and partial nerve ligation compared to wild-type mice, furthermore there was an increase in anti-inflammatory cytokines IL-4, IL-10 (Staton *et al,* 2008). Collectively these finding suggest that GPR55 modulation may be a target for inflammation and neuropathic pain.

6.1.3 GPR18 Receptor Agonist: NAGly

NAGly is a full agonist at GPR18 as shown by MAPK induction in HEC-1B GPR18 transfected cells. These effects were antagonised by pertussis toxin, suggesting G_i coupling (McHugh *et al,* 2012). NAGly is formed by the metabolism of AEA by FAAH (Burstein *et al,* 2000). Interestingly, AEA is a full agonist at both CB1 and CB2 receptors (Burstein and Zurier 2009) however; NAGly has no activity at either CB1 or CB2 receptors (Sheskin *et al,* 1997). Studies have suggested that NAGly may display anti-inflammatory properties via the reduction of pro-inflammatory macrophages (McHugh 2012) however the role of GPR18 in chondrocyte metabolism is unknown.

6.1.4 TRPV1 Receptor Agonist: OLDA

N-oleoyldopamine (OLDA) is an endogenous selective agonist at TRPV1 and *In vivo,* has been shown to have negative effects via inducing hyperalgesia (Chu *et al,* 2003). Activation of TRPV1 with vanilloid agonist capsaicin, induced the secretion of IL-6 from OA and RA synovial fibroblasts; these effects were attenuated by incubation with TRPV1 antagonist capsazepine (Engler *et al,* 2007). In contrast, activation of TRPV1 with cannabinoid ligand CBD induced anti-inflammatory effects in a rat model of acute inflammation (Costa *et al,* 2004). Together these findings suggest activation of TRPV1 may induce distinct responses depending on the agonist utilised.

6.1.5 PPARα Receptor Agonist: WY14643

Wy14643 is a selective PPARα agonist (Willson *et al,* 2000) and is thought to display anti-inflammatory properties mediated by the activation of PPARα (Clockaerts *et al,* 2011; Yoo *et al,* 2013). *In vivo,* Wy14643 attenuated LPS induced infiltration of inflammatory cells in a mouse model of acute lung injury, effects that were not seen in PPARα knockout mice (Yoo *et al,* 2013). Furthermore in human OA cartilage explants, Wy14643 reduced IL-1β induced MMP-1, -3 and -13 mRNA expression and the release of GAGs, NO and PGE_2, suggesting that activation of PPARα may be a therapeutic target for OA via the inhibition of catabolic processes (Clockaerts *et al,* 2011).

6.1.6 PPARδ Receptor Agonst: GW0742

Finally, GW0742 is a selective PPARδ agonist which displays over 1000 fold selectivity over PPARα and γ receptors (Sznaidman *et al,* 2003). *In vivo,*

GW0742 was shown to have anti-inflammatory effects in mouse models of acute lung injury or inflammation via the reduction of pro-inflammatory mediators (Di Paola *et al,* 2010;Galuppo *et al,* 2010). However, little is known regarding the effects of PPARδ activation on chondrocyte metabolism, although activation of this receptor is known to increase the expression of IL-1Ra in rat synovial fibroblasts (Moulin *et al,* 2005), suggesting it may have anti-arthritic properties.

6.1.7 PPARγ Receptor Agonists: Rosiglitazone and Troglitazone

Rosiglitazone and troglitazone are anti-diabetic drugs belonging to the drug class thiazolidinediones, and are both agonists at PPARγ (Murphy and Holder 2000). In rabbit articular chondrocytes rosiglitazone reduced IL-1β stimulated expression of MMP-1, -3 and -13 (Francois *et al,* 2004). Furthermore, rosiglitazone was shown to inhibit IL-1β induced MMP-1 expression in human synovial fibroblasts (Fahmi *et al,* 2002). Troglitazone inhibited the production of TNFα, IL-6, IL-8 and MMP-3 in RA fibroblast-like synovial cells (Yamasaki *et al,* 2002). Together these findings suggest that activation of PPARγ may be of therapeutic value in the treatment of OA, by reducing both inflammatory and destructive pathways.

6.1.8 WIN-55

WIN-55 is known to activate both CB1 and CB2 receptors, with Kis of 1.89-123 nM and 0.28-16.2 nM respectively (Pertwee *et al,* 2010). In addition, WIN-55 has been shown to bind to and increase the transcriptional activity of both PPARα and γ (O'Sullivan and Kendall 2010; Sun *et al,* 2006). Currently there is no reported evidence that WIN-55 activates PPARδ, however WIN-55 significantly induced the mRNA expression of PPARδ in human OA chondrocytes (Chapter 4, section 5.5.3.2). WIN-55 is also known to desensitize TRPV1, suggesting that WIN-55 binds to TRPV1 and attenuates its biological actions (Jeske *et al,* 2006). Finally, WIN-55 is thought to display no activity at GPR55 as would be shown by the formation of β-arrestin-GPR55 complex and ERK1/ERK2 phosphorylation (Kapur *et al,* 2009) and the effects of WIN-55 at GPR18 have yet to be determined. Although WIN-55 is known to activate certain cannabinoid receptors, it has been suggested that WIN-55 may mediated its effects via a non-cannabinoid receptor mechanism or a yet unidentified receptor (Selvi *et al,* 2008; Curran *et al,* 2005; Tauber *et al,* 2012).

6.2 Aims and Objectives

Aim: To determine the receptors responsible for the inhibition of IL-1β mediated MMP production and IL-1β signalling, a potential means by which cannabinoids may exert chondroprotective effects

Objectives:

- To investigate the effects of cannabinoid receptor agonists on IL-1β induced mRNA expression of MMP-3 and -13 in human OA chondrocytes.
- To investigate the effects of a combination of cannabinoid receptor agonists on IL-1β induced mRNA expression of MMP-3 and -13 in human OA chondrocytes.
- To determine the effects of cannabinoid receptor agonists used in combination on IL-1β induced phosphorylation of c-Jun, p38, ERK1/ERK2 and IκB in human OA chondrocytes.

6.3 Experimental Design

The effects of specific cannabinoid receptor agonists on IL-1β induced MMP-3 and MMP-13 mRNA expression and signalling pathways in chondrocytes obtained from human OA cartilage were investigated. Cartilage tissue was graded macroscopically 0-4 using the Outerbridge classification (Cameron *et al,* 2003). Chondrocytes were isolated from grade 2 or 3 cartilage tissue as representative of low degenerate and intermediate degenerate cartilage tissue. Cartilage from grade 4, severe degenerate tissue, was not used in the study, as the cell yield obtained was not sufficient. For investigation of mRNA expression, chondrocytes were expanded in monolayer to passage 2 and stimulated with 10 ng/ml IL-1β to induce the expression of MMP-3 and -13. Chondrocytes were treated with cannabinoid receptor agonists individually or in combination, both with and without 10 ng/ml IL-1β stimulation. Real-time PCR was used to investigate the effects of cannabinoid receptor agonists on MMP-3 and -13 mRNA expression. For signalling pathway investigation chondrocytes were expanded in monolayer to passage 2 and pre-treated with cannabinoid receptor

agonists in different combinations with and without 10-ng/ml IL-1β stimulation. Cell based ELISAs were used to measure the phosphorylation of cell signalling molecules p38, ERK1/ERK2, IκB and c-Jun.

6.4 Methodology

6.4.1 Human OA Cartilage Samples

Primary Human chondrocytes were obtained from articular cartilage removed from patients with symptomatic OA at the time of total knee replacement as described in section 2.4.1.

6.4.2 Macroscopic Grading of Cartilage Tissue

Cartilage tissue was macroscopically graded 0-4 using the Outerbridge Classification at the time of surgery prior to isolation of chondrocytes (Cameron *et al,* 2003) as described in section 2.4.2

6.4.3 Isolation of human chondrocytes

Human chondrocytes were isolated from cartilage as described in section 2.4.3.

6.4.4 OA Patient Samples

Chondrocyte cultures were derived from OA patient samples of macroscopic grades 2 or 3 (Table 6.1). Full patient sample information can be found in Appendix 1.

Analysis Performed	**Monolayer Cultures** **Macroscopic grade 2 or 3**
MMP-3 and -13 mRNA expression	HC5(1)*, HC21(5), HC23(4)*
Cell based ELISA	HC21(4)
MTS Cell Viability Assay	HC21(4), HC23(4)

Table 6.1 The patient samples used for each analysis performed on chondrocytes obtained from macroscopic grade 2 and 3 OA cartilage. *Indicates samples used for agonist combination treatments. Full patient details can be found in Appendix 1

6.4.5 Cannabinoid Receptor Agonist Concentrations

The concentration of cannabinoid receptor agonists tested can be seen in Table (6.2). Agonists were used at concentrations that would retain receptor selectivity and did not affect chondrocyte viability.

6.4.6 Cannabinoid Receptor Agonist Treatments

Cells were cultured in monolayer until 80% confluent before passaging as described in section 2.4.5. Chondrocytes were seeded in 6 well culture plates at a cell density of $5x10^5$ cells per well as described in section 2.4.6. Cells were treated with a range of receptor agonists both alone and in combination with 10 ng/ml IL-1β for 48 hours (Table 6.2).

6.4.7 Cannabinoid Receptor Agonist Combination Treatments

Cells were cultured in monolayer until 80% confluent before passaging as described in section 2.3.5. Chondrocytes were seeded in 6 well culture plates at a cell density of $5x10^5$ cells per well as described in section 2.4.6. Cells were treated with agonists for CB1 and CB2 followed by the addition of PPARα, δ or γ both with and without 10ng/ml IL-1β stimulation for 48 hours (Table 6.2). Cells were also treated with CB1, CB2, PPARα, δ or γ agonists in combinations both with and without of 10 ng/ml IL-1β for 48 hours (Table 6.2).

6.4.8 Cytotoxicity Studies MTS Assay

The effects of agonist treatments both alone and in combination on chondrocyte cell viability was performed as outlined section 2.4.12.

6.4.9 WIN-55 cell treatment

Since WIN-55 was shown to inhibit IL-1β induced MMP-3 and -13 mRNA expression (Chapter 2), WIN-55 was used as a positive control for MMP-3 and -13 mRNA expression. Cells were cultured in monolayer until 80% confluent before passaging as described in section 2.4.5. Chondrocytes were seeded in 6 well culture plates at a cell density of $5x10^5$ cells per well as described in section 2.3.6. Cells were treated as outlined in section 2.4.7.

6.4.10 RNA Extraction from Cells Cultured in Monolayer

Isolation of RNA was performed as described in section 2.4.13.

6.4.11 Reverse Transcription and Real-time PCR

RNA was reversed transcribed to cDNA as described in section 2.4.16. Taqman PCR was performed on cDNA as described in section 2.4.18. using pre-designed Taqman Gene Expression Assays (Table 2.4, Life Technologies).

6.4.12 Real-time PCR Analysis

Real-time PCR data was analysed using the $2^{-\Delta\Delta CT}$ (Livak and Schmittgen 2001) as described in section 2.4.2

6.4.13 Cell Based ELISA Agonist Treatments

Cells were cultured in monolayer until 80% confluent before passaging as described in section 2.4.5. Chondrocytes were seeded in a 96 well culture plate at a cell density of $1x10^5$ per well as outlined in section 4.3.9. Since a pre-treatment of WIN-55 was required for the inhibition of IL-1β induced phosphorylation of ERK1/ERK2, c-Jun, and IκB (Chapter 4), cells were pre-treated for 48 hours with different combinations of receptor agonists alone or in the presence of 10 ng/ml IL-1β stimulation for the last 30 minutes of agonist treatment (Table 6.2).

6.4.14 Cell Based ELISA

Cell based ELISA's were performed at described in section 4.3.10.

6.4.15 Statistical Analysis

Statistical analysis was performed as described in section 2.5.

Cannabinoid receptor	Agonist	EC50 /K_i Value	Experimental Concentration Range for mRNA expression (+/- 10 ng/ml IL-1β)	Combination Treatments for mRNA expression and cell based ELISA (+/- 10 ng/ml IL-1β).
CB1	ACEA (Tocris)	1.4 nM	0.2, 2 and 20 μM	2 or 20 μM
CB2	HU308 (Tocris)	5.57 nM	0.1, 1 and 10 μM	1 or 10 μM
GPR55	LPI (Sigma-Aldrich)	1.2 μM	0.1, 1 and 10 μM	Not Investigated
GPR18	NAGly (Tocris)	44.5 nM	0.3, 3 and 30 μM	Not Investigated
TRPV1	OLDA (Tocris)	36 nM	0.03, 0.3 and 3 μM	Not Investigated
PPARα	Wy-14643 (Tocris)	5 μM	1, 10 and 100 μM	10 or 100 μM
PPARδ	GW0742 (Tocris)	0.001 μM	0.02, 0.2 and 2 μM	0.2 or 2 μM
PPARγ	Troglitazone (Tocris) Rosiglitazone (Cayman Chemical)	555 nM 43 nM	0.1, 1 and 10 μM 10 and 100 μM	1 or 10 μM 10 and 100 μM

Table 6.2 Cannabinoid Receptor Selective Agonists. Agonists were tested individually using a range of concentrations both with and without IL-1β stimulation for 48 hours for analysis of MMP-3 and -13 mRNA expression. CB1, CB2, PPARα, δ and γ were used in combination at a lower concentration and a 10 fold higher concentration with and without IL-1β stimulation for mRNA expression of MMP-3 and -13 and cell-based ELISA analysis.

6.5 Results

6.5.1 Cell Viability MTS assay

No effects on cell viability following treatments with ACEA at a concentration of 0.002 to 20 μM, HU308 at a concentration of 0.005 to 50 μM, LPI at a concentration of 0.1 to 10 μM, NAGly at a concentration of 5 to 30 μM, OLDA at a concentration of 0.03 to 30 μM, Wy14643 at a concentration of 0.5 to 250 μM, GW0742 at a concentration 0.001 to 10 μM, troglitazone at a concentration of 0.5 to 10 μM and rosiglitazone at a concentration of 10 to 100 μM for 48 hours was observed (Data not shown).

Combination treatments of 20 μM ACEA and 10 μM HU308 together or with the addition of 100 μM Wy14643, 10 μM troglitazone or 100 μM rosiglitazone for 48 hours had no effect on cell viability (Figure 6.1). All the agonists used in combination at a lower concentration of 2 μM ACEA, 1 μM HU308, 10 μM Wy14643, 1 μM troglitazone and 10 μM rosiglitazone for 48 hours had no effect on cell viability (Figure 6.1). A combination of all the agonists at the higher concentration of 20 μM ACEA, 10 μM HU308, 100 μM Wy14643, 10 μM troglitazone and 100 μM rosiglitazone for 48 hours reduced cell viability to 71 % compared to untreated control cells ($p<0.05$) (Figure 6.1). All concentrations of agonists were tested in combination with IL-1β and no significant effect on cell viability was observed for individual agonists, however combination of all the agonists at a higher concentration of 20 μM ACEA, 10 μM HU308, 100 μM Wy14643, 10 μM troglitazone and 100 μM rosiglitazone in combination with IL-1β for 48 hours reduced cell viability to 80 % compared to untreated control cells ($p<0.05$) (Data not shown).

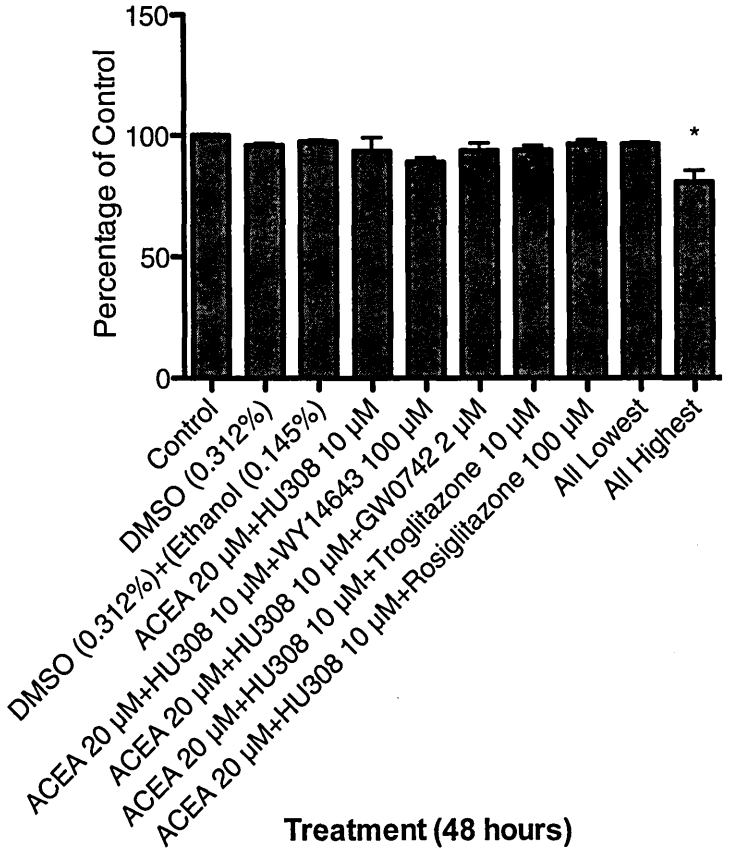

Figure 6.1 MTS Cell Viability Assay. Combination treatments of 20 µM ACEA and 10 µM HU308 together or with the addition of 100 µM Wy14643, 10 µM troglitazone or 100 µM rosiglitazone for 48 hours had no effect on cell viability. All agonists used in combination at a lower concentration of 2 µM ACEA, 1 µM HU308, 10 µM Wy14643, 1 µM troglitazone and 10 µM rosiglitazone for 48 hours had no effect on cell viability. A combination of all agonists at a higher concentration of 20 µM ACEA, 10 µM HU308, 100 µM Wy14643, 10 µM troglitazone and 100 µM rosiglitazone for 48 hours significantly reduced cell viability compared to untreated control cells. Data represents mean percentage of control under treatment conditions ± SEM.*$p<0.05$ compared to untreated control cells. n=3 obtained from patient sample (HC23(4)).

6.5.2 Individual Cannabinoid Receptor Agonists

6.5.2.1 Effects of IL-1β on MMP-3 and -13 mRNA expression

Following IL-1β stimulation for 48 hours there was a significant increase in MMP-3 and -13 expression compared to DMSO or ethanol control ($p<0.001$) (Figure 6.2-6.10).

6.5.2.2 GPCR Agonist Effects on IL-1β induced MMP-3 and -13 mRNA Expression

IL-1β induced MMP-3 and -13 mRNA expression was not significantly affected by any dose of CB1 agonist (ACEA) (Figure 6.2), CB2 agonist (HU308) (Figure 5.3), GPR55 agonist (LPI) (Figure 6.4) or GPR18 agonist (NAGly) (Figure 6.5) ($p<0.05$) compared to IL-1β stimulation alone ($p>0.05$) for 48 hours. Treatment of 10 μM CB2 (HU308) agonist in combination with IL-1β has a slight stimulatory effect on MMP-13 expression compared to IL-1β treatment alone, however this was not significant (Figure 6.3).

6.5.2.3 TRPV1 Agonist Effects on IL-1β induced MMP-3 and -13 mRNA Expression

IL-1β induced MMP-3 and -13 mRNA expression was not significantly affected by any dose of TRPV1 agonist (OLDA) compared to IL-1β stimulation alone ($p>0.05$) (Figure 6.6). However, OLDA treatment at a concentration of 0.3 and 3 μM in combination with IL-1β further increased MMP-13 mRNA expression compared to IL-1β ($p<0.05$ and $p<0.001$) (Figure 6.6).

6.5.2.4 PPAR Agonist Effects on IL-1β induced MMP-3 and -13 mRNA Expression

IL-1β induced MMP-3 and -13 mRNA expression was not significantly decreased by any dose of PPARα agonist (Wy14643) (Figure 6.7), PPARδ agonist (GW0742) (Figure 6.8) or PPARγ agonists (troglitazone or rosiglitazone (Figure 6.9 & 6.10) compared to IL-1β stimulation alone ($p>0.05$). However, PPARα agonist (Wy14643) treatment at a concentration of 100 μM in combination with IL-1β further increased MMP-13 mRNA expression compared to IL-1β stimulation alone ($p<0.01$) (Figure 6.7).

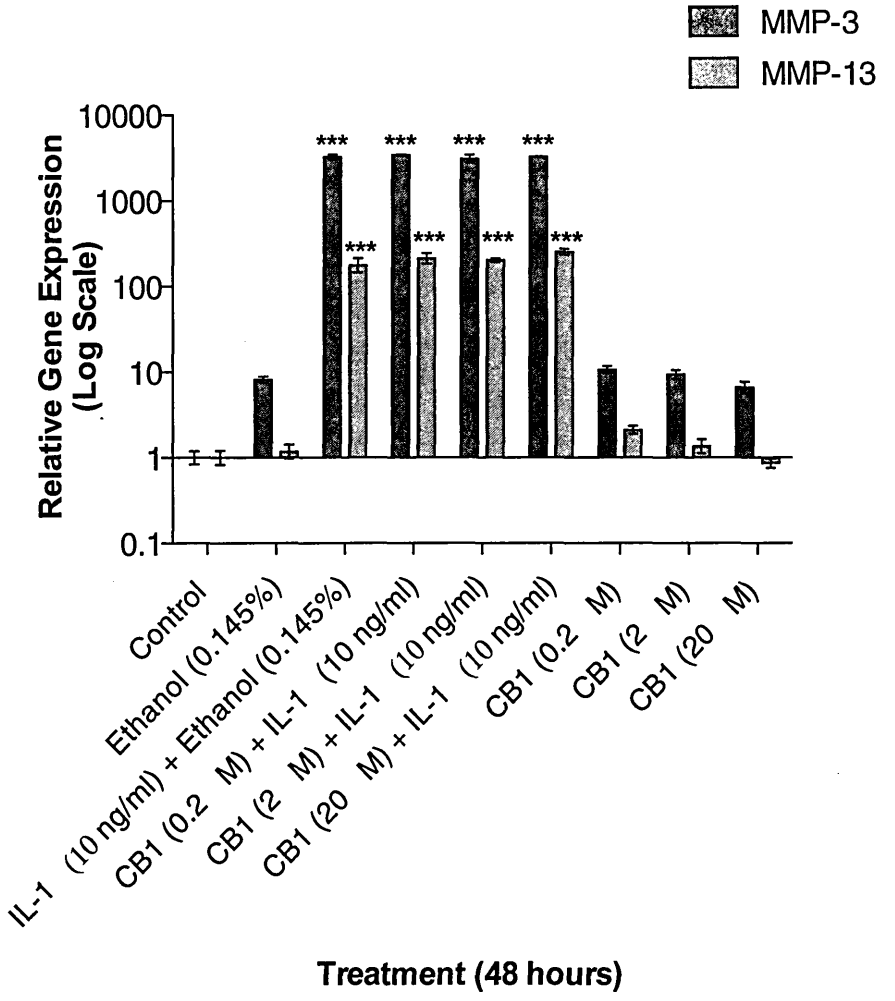

Figure 6.2 The effects of CB1 receptor agonist ACEA on MMP-3 and MMP-13 mRNA expression in human OA chondrocytes cultured in monolayer. IL-1β stimulation both alone and in combination with 0.2, 2 and 20 μM the CB1 agonist (ACEA) induced MMP-3 and -13 mRNA expression. 0.2, 2 and 20 μM CB1 agonist (ACEA) treatment alone had no effect on MMP-3 or -13 mRNA expression. Data represents mean fold change of mRNA expression normalised to internal reference gene and untreated control ± SEM. **p<0.01 and ***p<0.001 compared to solvent control. n=3 obtained from one macroscopic grade 3 patient sample (HC21(5)).

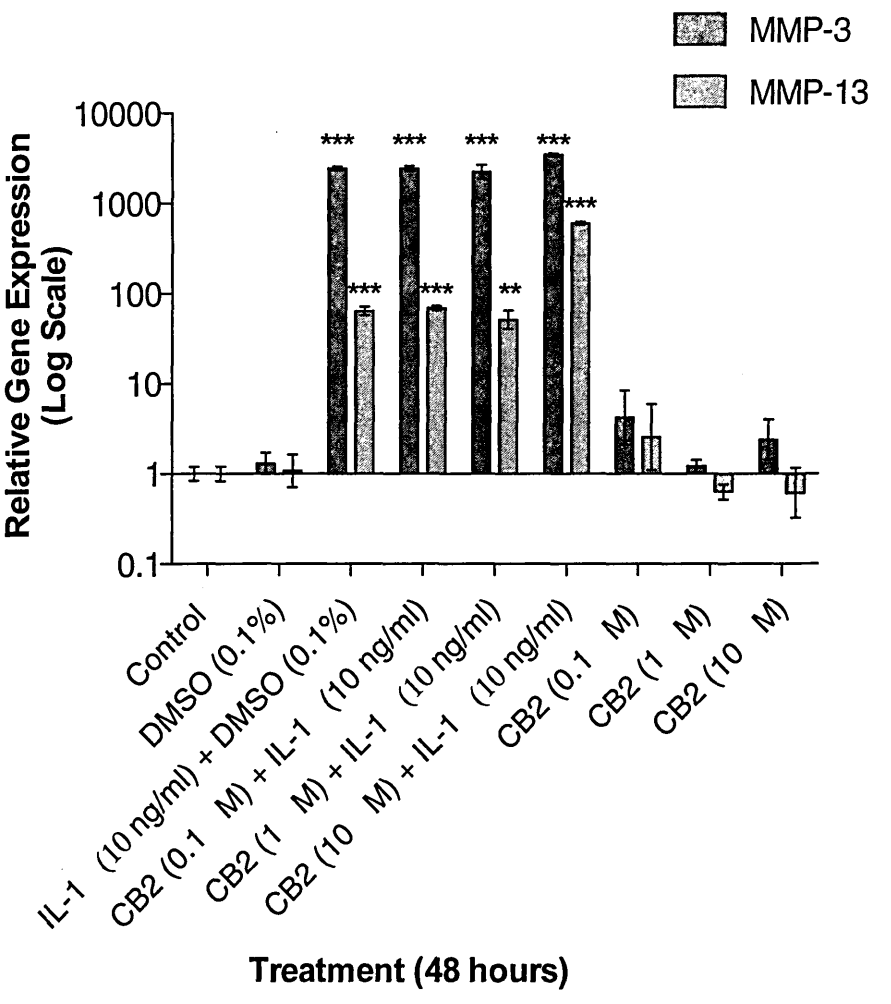

Figure 6.3 The effects of CB2 receptor agonist HU308 on MMP-3 and MMP-13 mRNA expression in human OA chondrocytes cultured in monolayer. IL-1β stimulation both alone and in combination with 0.1, 1 and 10 μM the CB1 agonist (HU308) induced MMP-3 and -13 mRNA expression. 0.1, 1 and 10 μM CB2 agonist (HU308) treatment alone had no effect on MMP-3 or -13 mRNA expression. Data represents mean fold change of mRNA expression normalised to internal reference gene and untreated control ± SEM. **p<0.01 and ***p<0.001 compared to solvent control. n=3 obtained from one macroscopic grade 3 patient sample (HC21(5)).

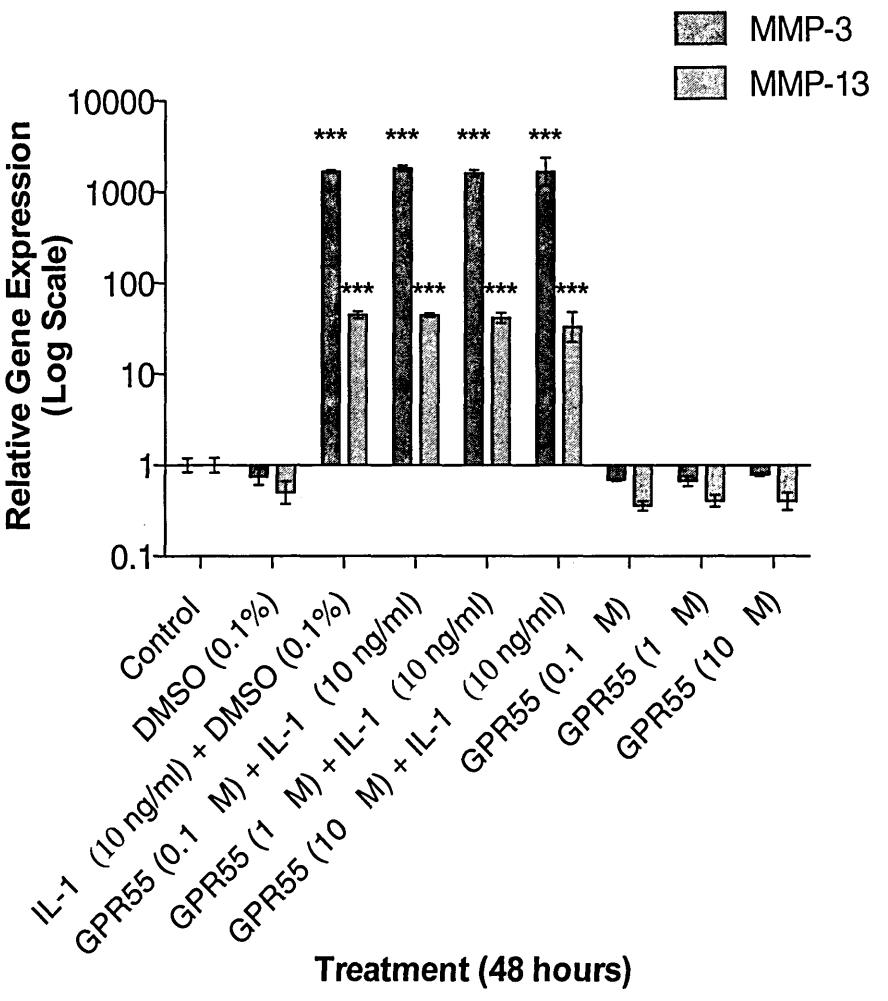

Figure 6.4 The effects of GPR55 receptor agonist LPI on MMP-3 and MMP-13 mRNA expression in human OA chondrocytes cultured in monolayer. IL-1β stimulation both alone and in combination with 0.1, 1 and 10 μM with GPR55 agonist (LPI) induced MMP-3 and -13 mRNA expression. 0.1, 1 and 10 μM GPR55 agonist (LPI) treatment alone had no effect on MMP-3 or -13 mRNA expression. Data represents mean fold change of mRNA expression normalised to internal reference gene and untreated control ± SEM. **p<0.01 and ***p<0.001 compared to DMSO control. n=3 obtained from one macroscopic grade 3 patient sample (HC21(5)).

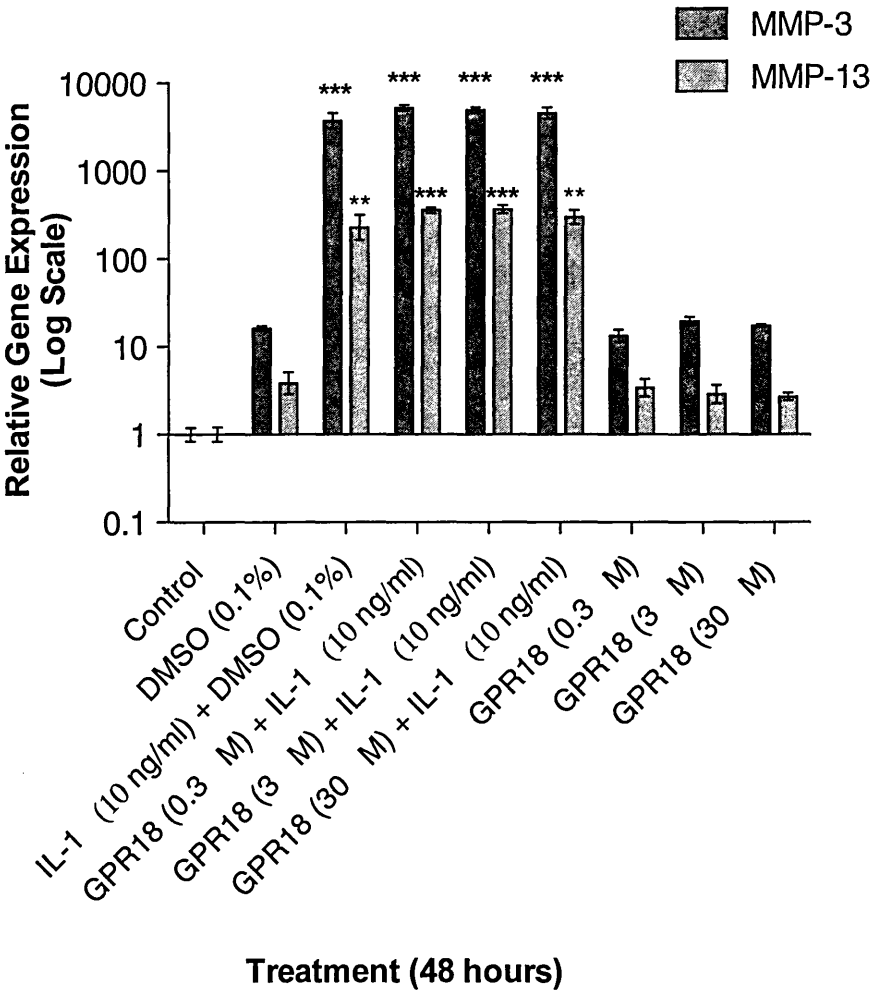

Figure 6.5 The effects of GPR18 receptor agonist NAGly on MMP-3 and MMP-13 mRNA expression in human OA chondrocytes cultured in monolayer. IL-1β stimulation both alone and in combination with 0.3, 3 and 30 μM GPR18 agonist (NAGly) induced MMP-3 and -13 mRNA expression. 0.3, 3 and 30 μM GPR18 (NAGly) treatment alone had not effect on MMP-3 or -13 mRNA expression. Data represents mean fold change of mRNA expression normalised to internal reference gene and untreated control ± SEM. **p<0.01 and ***p<0.001 compared to DMSO control. n=3 obtained from one macroscopic grade 3 patient sample (HC21(5)).

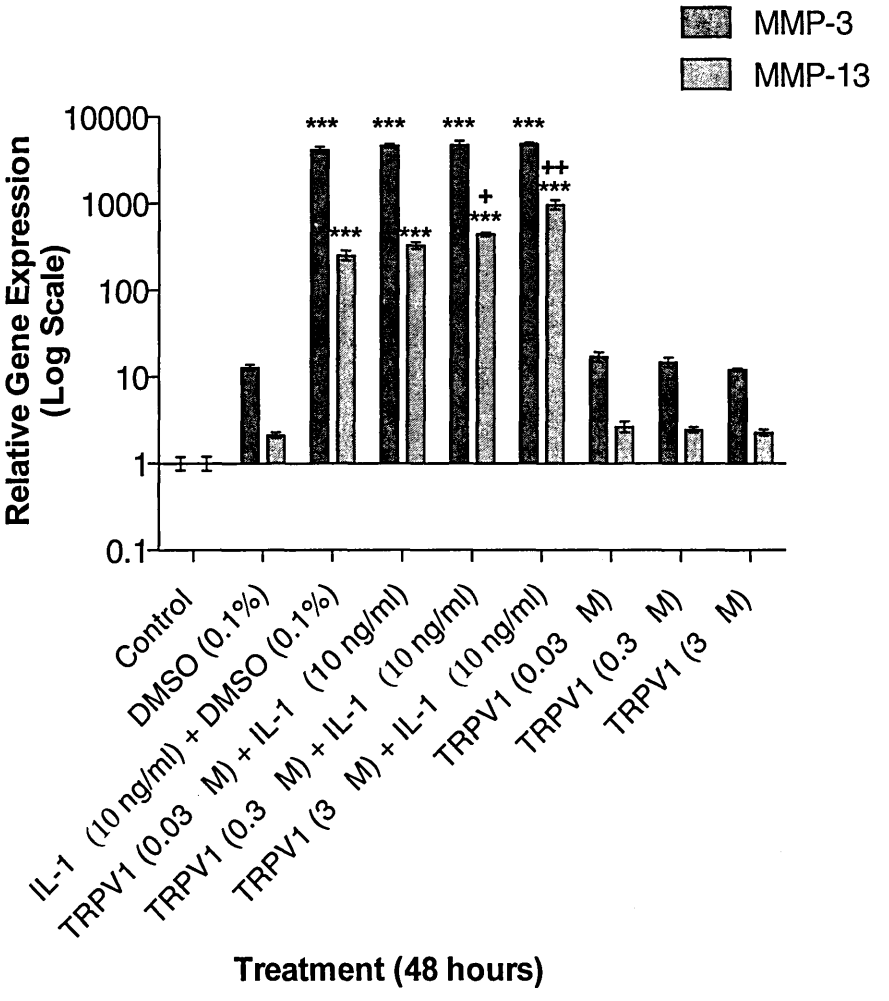

Figure 6.6 The effects of TRPV1 receptor agonist OLDA on MMP-3 and MMP-13 mRNA expression in human OA chondrocytes cultured in monolayer. IL-1β stimulation both alone and in combination with 0.03, 0.3 and 3 μM OLDA increased MMP-3 and -13 mRNA expression. TRPV1 agonist (OLDA) treatment at a concentration of 0.3 and 3 μM in combination with IL-1β increased MMP-13 expression compared to IL-1β treatment alone. TRPV1 agonist (OLDA) treatment at a concentration of 0.03, 0.3 and 3 μM alone had no effect on MMP-3 or -13 mRNA expression. Data represents mean fold change of mRNA expression normalised to internal reference gene and untreated control ± SEM. ***$p<0.001$ compared to DMSO control and +$p<0.05$, ++0.01 compared to IL-1β treatment alone. n=3 obtained from one macroscopic grade 3 patient sample (HC21(5)).

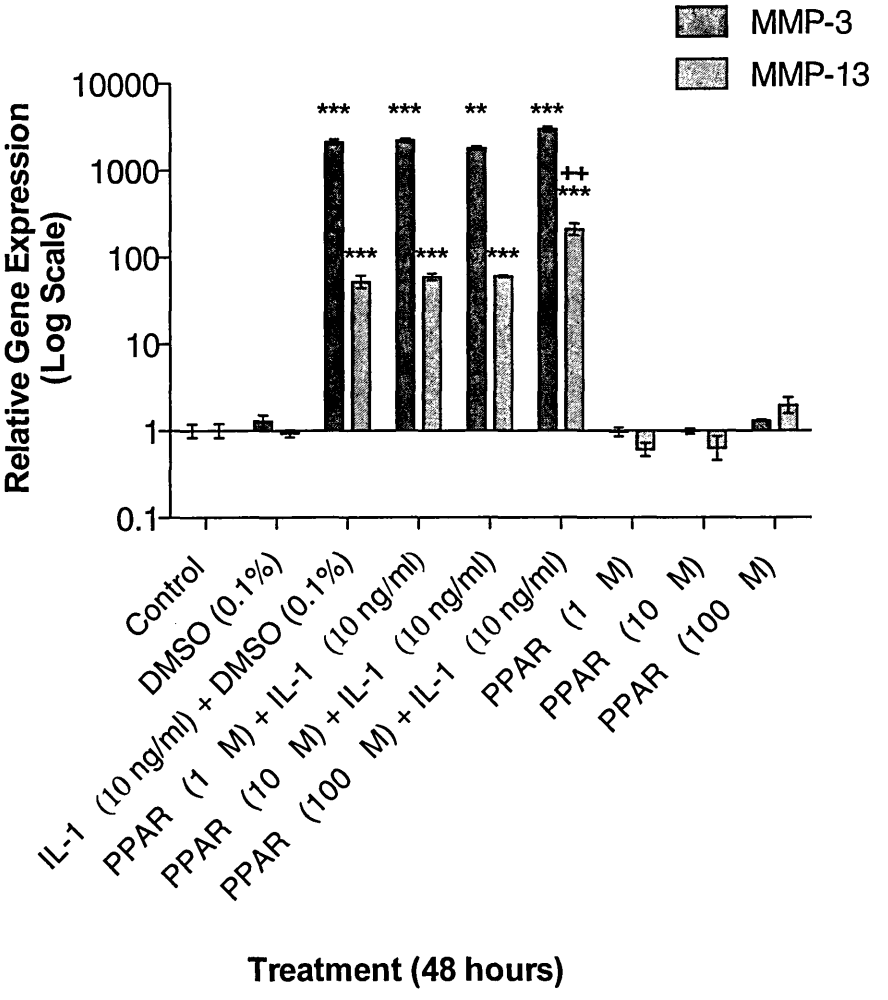

Figure 6.7 The effects of PPARα receptor agonist Wy14643 on MMP-3 and MMP-13 mRNA expression in human OA chondrocytes cultured in monolayer. IL-1β stimulation both alone and in combination with 1, 10 and 100 μM PPARα agonist (Wy14643) induced MMP-3 and -13 mRNA expression. PPARα agonist (Wy14643) treatment at a concentration of 100 μM in combination with IL-1β significantly induced MMP-13 expression compared to IL-1β treatment alone. PPARα (Wy14643) treatment at a concentration of 1, 10, and 100 μM alone had not effect on MMP-3 or -13 mRNA expression. Data represents mean fold change of mRNA expression normalised to internal reference gene and untreated control ± SEM. **p<0.01, ***p<0.001 compared to DMSO control and ++0.01 compared to IL-1β treatment alone. n=3 obtained from one macroscopic grade 3 patient sample (HC21(5)).

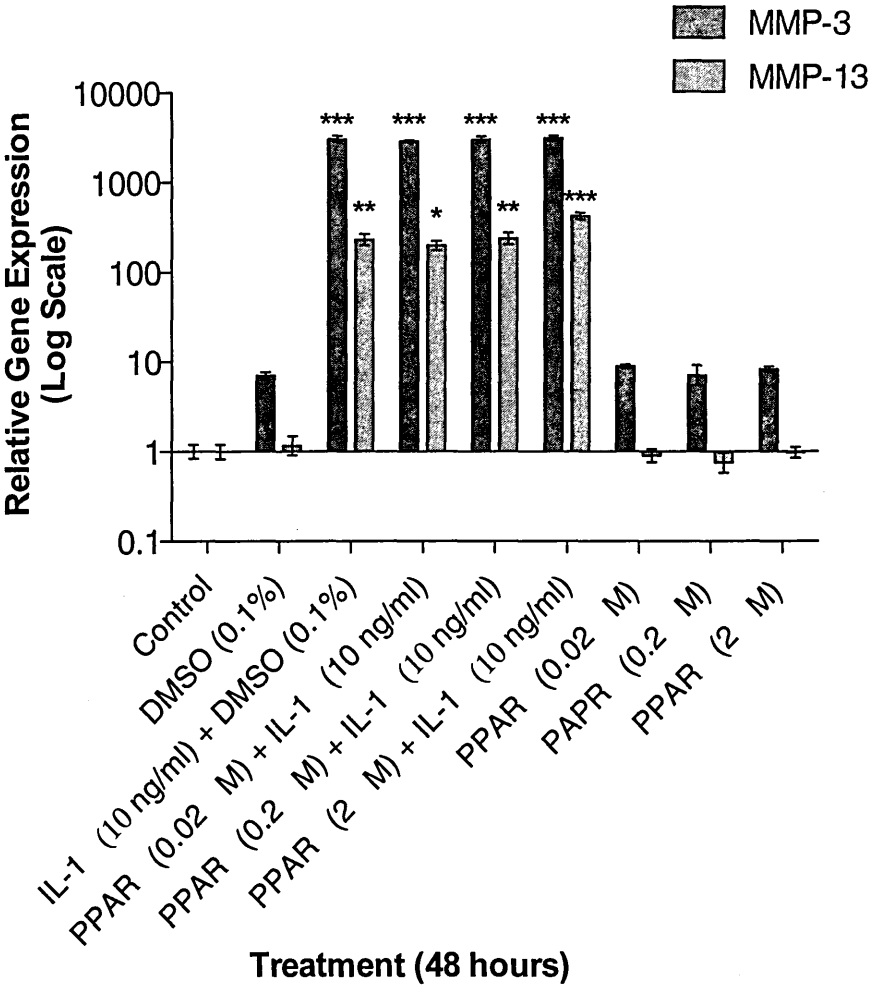

Figure 6.8 The effects of PPARδ receptor agonist GW0742 on MMP-3 and MMP-13 mRNA expression in human OA chondrocytes cultured in monolayer. IL-1β stimulation both alone and in combination with 0.02, 0.2 and 2 μM PPARδ agonist (GW0742) induced MMP-3 and -13 mRNA expression. PPARδ agonist (GW0742) treatment at a concentration of 0.02, 0.2, and 2 μM alone had no effect on MMP-3 or -13 mRNA expression. Data represents mean fold change of mRNA expression normalised to internal reference gene and untreated control ± SEM. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared to DMSO control. n=3 obtained from one macroscopic grade 3 patient sample (HC21(5)).

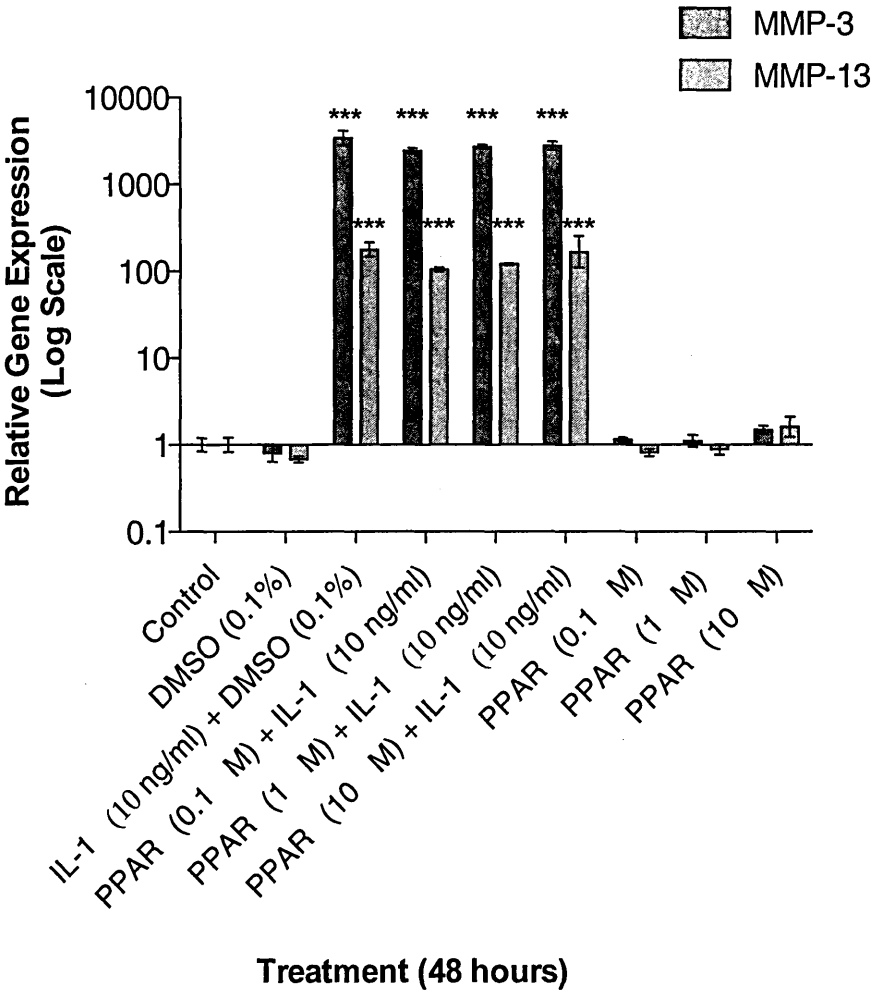

Figure 6.9 The effects of PPARγ receptor agonist troglitazone on MMP-3 and MMP-13 mRNA expression in human OA chondrocytes cultured in monolayer. IL-1β stimulation both alone and in combination with 0.1, 1 and 10 μM PPARγ agonist (troglitazone) induced MMP-3 and -13 mRNA expression. PPARγ agonist (troglitazone) treatment at a concentration of 0.1, 1 and 10 μM alone had no effect on MMP-3 or -13 mRNA expression. Data represents mean fold change of mRNA expression normalised to internal reference gene and untreated control ± SEM. ***$p<0.001$ compared to DMSO control. n=3 obtained from one macroscopic grade 3 patient sample (HC21(5)).

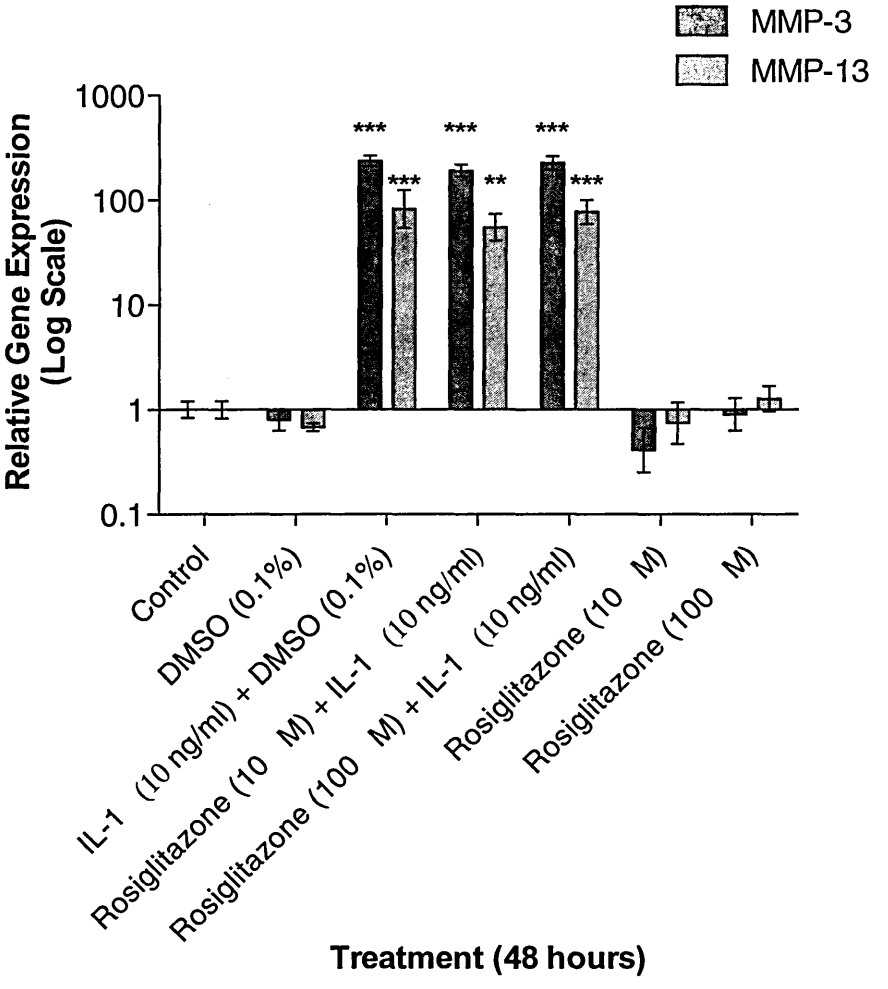

Figure 6.10 The concentration effects of PPARγ receptor agonist rosiglitazone on MMP-3 and MMP-13 mRNA expression in human OA chondrocytes cultured in monolayer. IL-1β stimulation both alone and in combination with 1 and 10 μM PPARγ agonist (rosiglitazone) induced MMP-3 and -13 mRNA expression. PPARγ agonist (rosiglitazone) treatment at a concentration of 1 and 10 μM alone had no effect on MMP-3 or -13 mRNA expression. Data represents mean fold change of mRNA expression normalised to internal reference gene and untreated control ± SEM. ***$p<0.001$ compared to DMSO control. n=3 obtained from one macroscopic grade 3 patient sample (HC21(5)).

6.5.3 Cannabinoid Receptor Agonists Combination Treatments.

Cannabinoid receptor agonists for CB1 (ACEA), CB2 (HU308), GPR55 (LPI), GPR18 (NAGly), TRPV1 (OLDA), PPARα (Wy14643), PPARδ (GW0742) and PPARγ (troglitazone or rosiglitazone) used individually failed to counteract IL-1β induction of MMP-3 and -13 mRNA expression. Thus, a combination of agonists for CB1, CB2 and PPARα, δ and γ were investigated including ACEA, HU308, Wy14643, GW0742, troglitazone and rosiglitazone (Table 6.2).

6.5.3.1 Effects of IL-1β on MMP-3 and -13 mRNA expression

Following IL-1β stimulation for 48 hours there was a significant increase in MMP-3 and -13 expression compared to DMSO and ethanol control ($p<0.001$) (Figure 6.11-6.15).

6.5.3.2 Effects of Receptor Agonists Combination Treatments

CB1 (ACEA) and CB2 (HU308) agonist treatment in combination with either PPARα agonist (Wy14643) PPARδ agonist (GW0742) or PPARγ agonists (troglitazone or rosiglitazone) with IL-1β for 48 hours failed to counteract the effects of IL-1β on both MMP-3 and -13 induction (Figure 6.11-6.15). CB1 (ACEA), CB2 (HU308) and PPARγ (troglitazone or rosiglitazone) agonist treatments in combination with IL-1β for 48 hours induced a non-significant reduction in MMP-13 mRNA expression compared to IL-1β stimulation alone (Figure 6.14 and 6.15). CB1 (ACEA) and CB2 (HU308) agonist treatment in combination with either PPARα agonist (Wy14643), PPARδ agonist (GW0742) or PPARγ agonists (troglitazone or rosiglitazone) for 48 hours had no significant effect on MMP-3 or -13 mRNA expression compared to DMSO control (Figure 6.11-6.15).

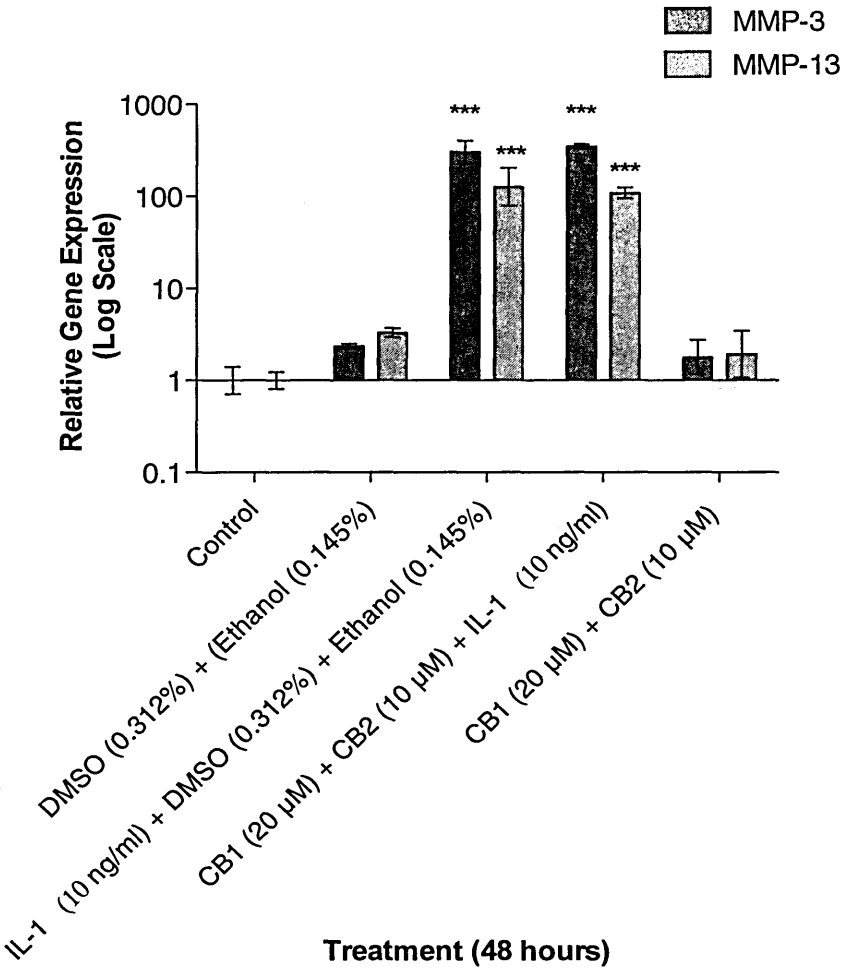

Figure 6.11 The effects of CB1 and CB2 receptor agonists ACEA and HU308 on MMP-3 and -13 mRNA expression in OA chondrocytes cultured in monolayer. IL-1β stimulation both alone and in combination with CB1 (ACEA) and CB2 (HU308) agonists significantly induced MMP-3 and -13 mRNA expression. CB1 (ACEA) and CB2 (HU308) agonist treatment in combination had no significant effect on MMP-3 or -13 mRNA expression. Data represents mean fold change of mRNA expression normalised to internal reference gene and untreated control ± SEM. ***$p<0.001$ compared to DMSO control. n=6 obtained from two patient samples.

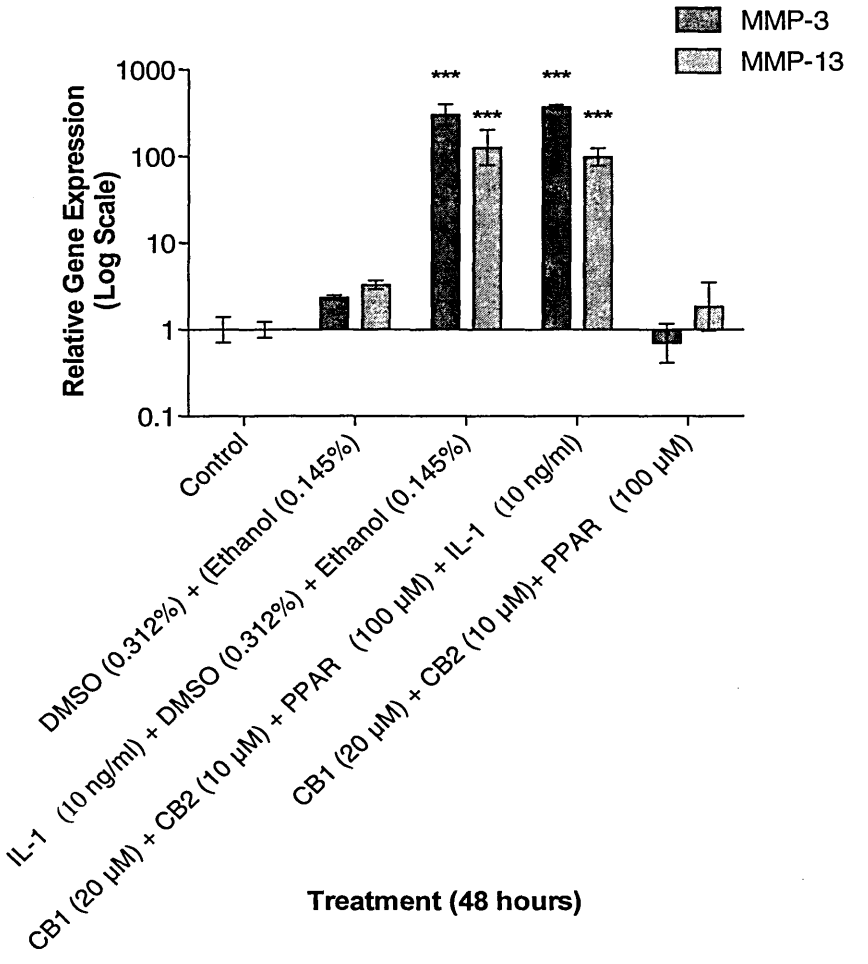

Figure 6.12 The effects of CB1, CB2 and PPARα receptor agonists ACEA, HU308 and Wy14643 on MMP-3 and -13 mRNA expression in OA chondrocytes cultured in monolayer. IL-1β stimulation both alone and in combination with CB1 (ACEA), CB2 (HU308) and PPARα (Wy14643) agonists significantly induced MMP-3 and -13 mRNA expression. CB1 (ACEA), CB2 (HU308) and PPARα (Wy14643) treatment in combination had no significant effect on MMP-3 or -13 mRNA expression. Data represents mean fold change of mRNA expression normalised to internal reference gene and untreated control ± SEM. ***$p<0.001$ compared to DMSO control. n=6 obtained from two patient samples.

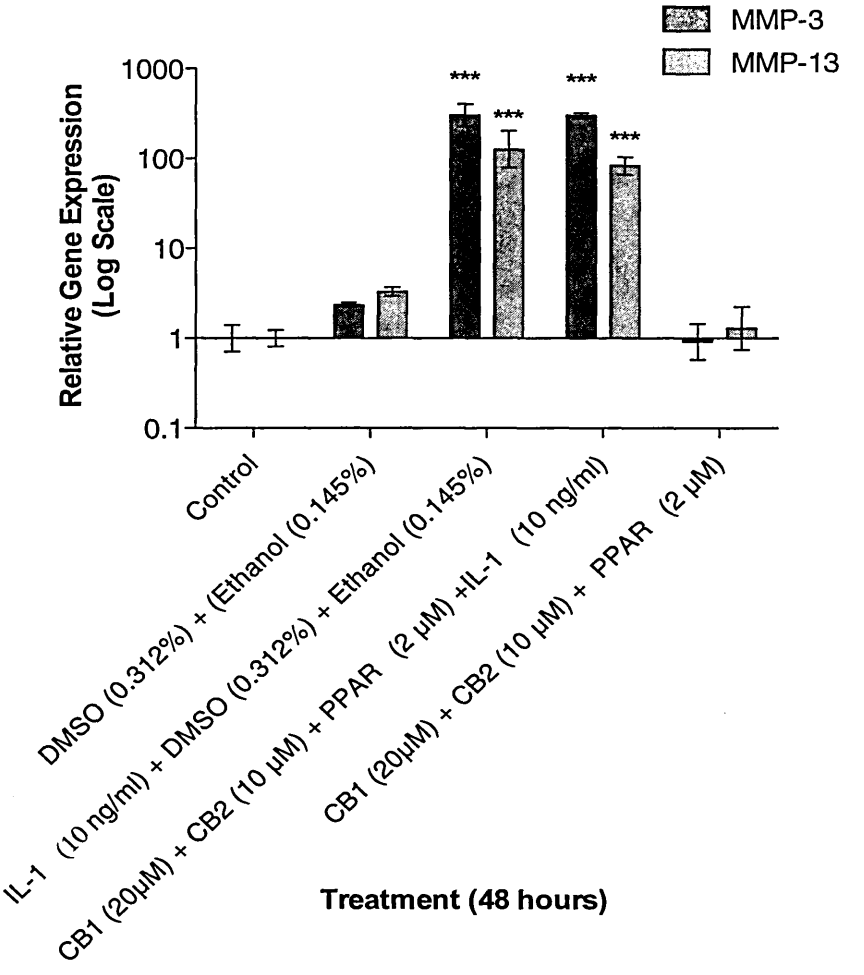

Figure 6.13 The effects of CB1, CB2 and PPARδ receptor agonists ACEA, HU308 and GW0742 on MMP-3 and -13 mRNA expression in OA chondrocytes cultured in monolayer. IL-1β stimulation both alone and in combination with CB1 (ACEA), CB2 (HU308) and PPARδ (GW0742) agonists significantly induced the expression of MMP-3 and -13 mRNA expression compared to the DMSO and ethanol control. CB1 (ACEA), CB2 (HU308) and PPARδ (GW0742) agonists treatments in combination had no significant effect on MMP-3 or -13 mRNA expression. Data represents mean fold change of mRNA expression normalised to internal reference gene and untreated control ± SEM. ***$p<0.001$ compared to DMSO and ethanol control. n=6 obtained from two patient samples.

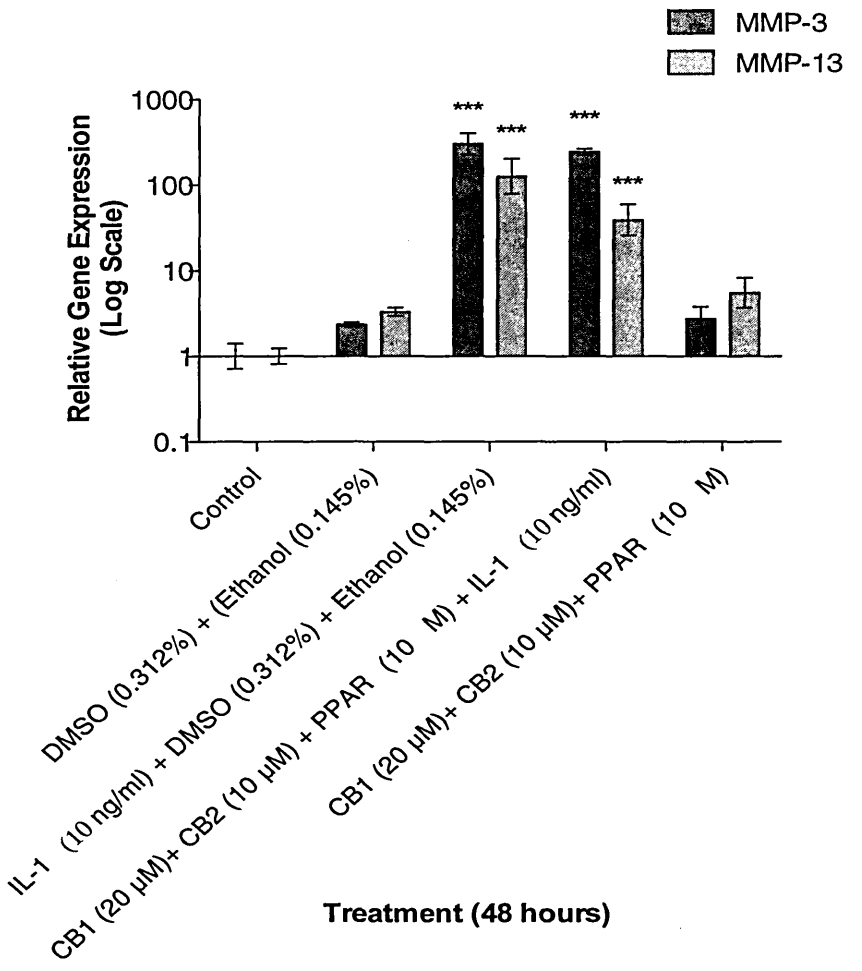

Figure 6.14 The effects of CB1, CB2 and PPARγ receptor agonists ACEA, HU308 and troglitazone on MMP-3 and -13 mRNA expression in OA chondrocytes cultured in monolayer. IL-1β stimulation both alone and in combination with CB1 (ACEA), CB2 (HU308) and PPARγ (troglitazone) agonists significantly induced the expression of MMP-3 and -13 mRNA expression compared to the DMSO and ethanol control. CB1 (ACEA), CB2 (HU308) and PPARγ (troglitazone) treatment in combination had no significant effect on MMP-3 or -13 mRNA expression. Data represents mean fold change of mRNA expression normalised to internal reference gene and untreated control ± SEM.***$p<0.001$ compared to DMSO and ethanol control. n=6 obtained from two patient samples.

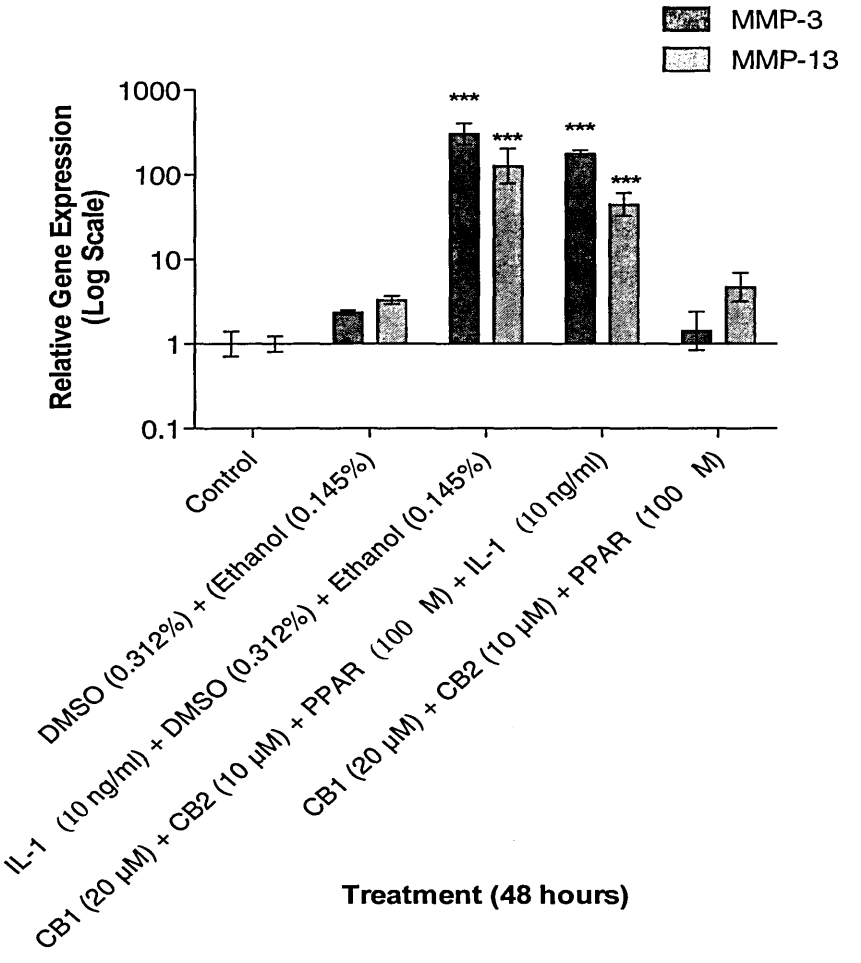

Figure 6.15 The effects of CB1, CB2 and PPARγ receptor agonists ACEA, HU308 and rosiglitazone on MMP-3 and -13 mRNA expression in OA chondrocytes cultured in monolayer. IL-1β stimulation both alone and in combination with CB1 (ACEA), CB2 (HU308) and PPARγ (rosiglitazone) agonists significantly induced the expression of MMP-3 and -13 mRNA expression compared to the DMSO and ethanol control. CB1 (ACEA), CB2 (HU308) and PPARγ (rosiglitazone) treatment in combination had no significant effect on MMP-3 or -13 mRNA expression. Data represents mean fold change of mRNA expression normalised to internal reference gene and untreated control ± SEM. ***p<0.001 compared to DMSO and ethanol control. n=6 obtained from two patient samples.

6.5.3.3 CB1 and CB2 Receptor Agonists and WIN-55 Combination Treatments

IL-1β stimulation for 48 hours significantly induced the mRNA expression of MMP-3 and -13 ($p<0.001$) (Figure 6.16). CB1 (ACEA), CB2 (HU308) and WIN-55 agonist treatment in combination with IL-1β for 48 hours significantly reduced MMP-3 and -13 mRNA expression compared to IL-1β stimulation alone and DMSO control ($p<0.001$) (Figure 6.16). CB1 (ACEA), CB2 (HU308) and WIN-55 agonist treatments alone for 48 hours significantly reduced MMP-3 and -13 mRNA expression below basal levels compared to DMSO control ($p<0.001$) (Figure 6.16). WIN-55 treatment in combination with IL-1β for 48 hours significantly reduced both MMP-3 and -13 mRNA expression compared to IL-1β stimulation alone and DMSO control ($p<0.001$) (Figure 6.16). WIN-55 treatment alone for 48 hours significantly reduced the MMP-3 mRNA expression ($p<0.001$) compared to DMSO control and abolished MMP-13 mRNA expression (Figure 6.16).

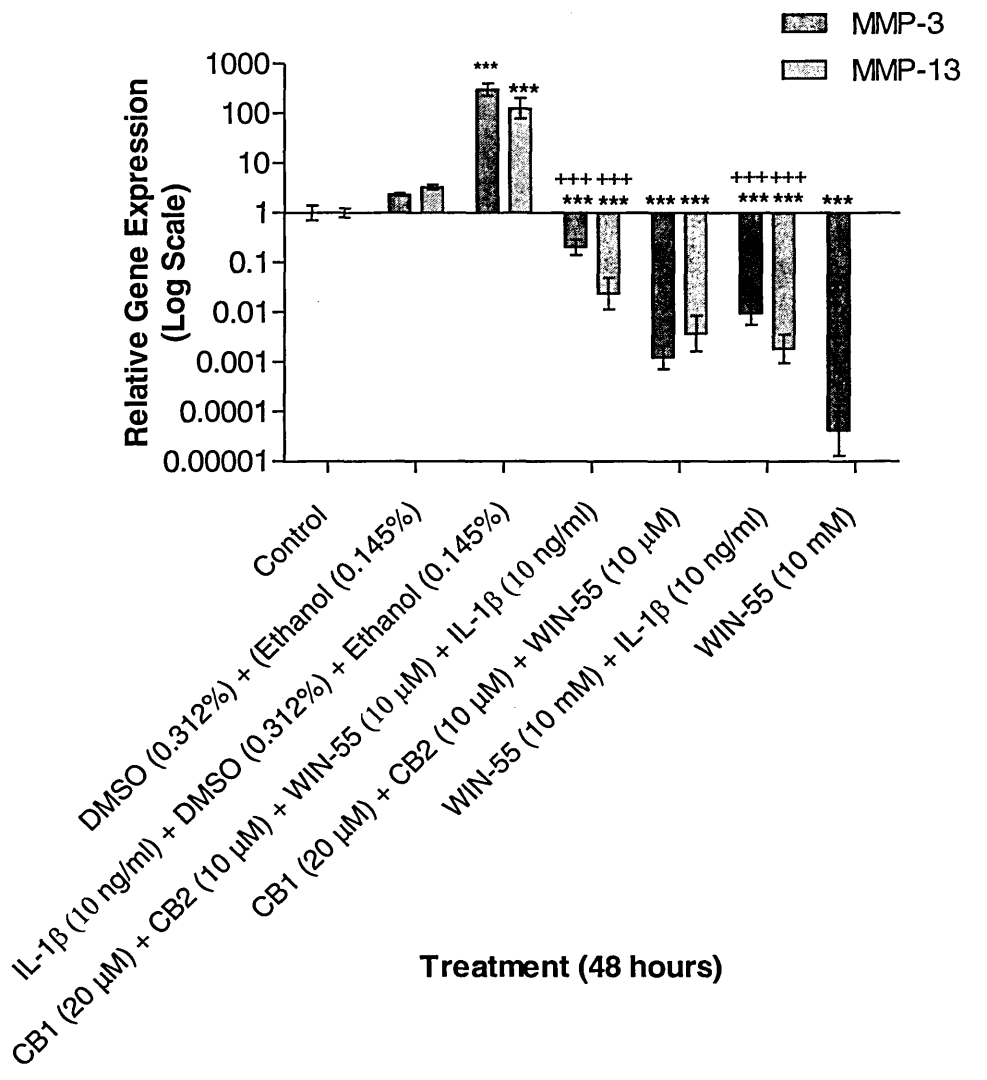

Figure 6.16 The effects of CB1, CB2 agonists ACEA, HU308 and WIN-55 on MMP-3 and -13 mRNA expression in OA chondrocytes cultured in monolayer. IL-1β stimulation induced MMP-3 and -13 mRNA expression. CB1 (ACEA), CB2 (HU308) and WIN-55 treatment in combination both alone and in the presence of IL-1β reduced of MMP-3 and -13 mRNA expression below basal levels. WIN-55 both alone and in combination with IL-1β reduced or abolished MMP-3 and -13 mRNA expression. Data represents mean fold change of mRNA expression normalised to internal reference gene and untreated control ± SEM. ***$p<0.001$ compared to DMSO control and +++$p<0.001$ compared to IL-1β stimulation. n=6 obtained from two patient samples.

6.5.3.4 CB1, CB2 and PPARα, δ and γ Receptor Agonist Combination Treatments.

IL-1β stimulation for 48 hours significantly induced the mRNA expression of MMP-3 and -13 ($p<0.001$) (Figure 6.18). All agonists combined at a lower concentration of 2 μM, 1 μM, 10 μM, 0.2 μM, 1 μM and 10 μM for CB1 (ACEA), CB2 (HU308), PPARα (Wy14643), PPARδ (GW0742) and PPARγ (troglitazone and rosiglitazone) respectively in combination with IL-1β for 48 hours did not counteract the effects of IL-1β on induction of MMP-3 and -13 mRNA expression ($p>0.05$) (Figure 6.17). All agonists at a higher concentration of 20 μM, 10 μM, 100 μM, 2 μM, 10 μM and 100 μM for CB1 (ACEA), CB2 (HU308), PPARα (Wy14643), PPARδ (GW0742) and PPARγ (troglitazone and rosiglitazone) respectively in combination with IL-1β for 48 hours significantly reduced the expression of both MMP-3 ($p<0.01$) and -13 ($p<0.001$) mRNA expression compared to IL-1β stimulation alone (Figure 6.18). Following treatment with all agonists at lower concentrations for 48 hours there was no significant effect on MMP-3 or -13 mRNA expression, however treatment with agonists at higher concentrations for 48 hours resulted in a significant decrease in both MMP-3 and MMP-13 mRNA expression compared to DMSO control ($p<0.001$) (Figure 6.18).

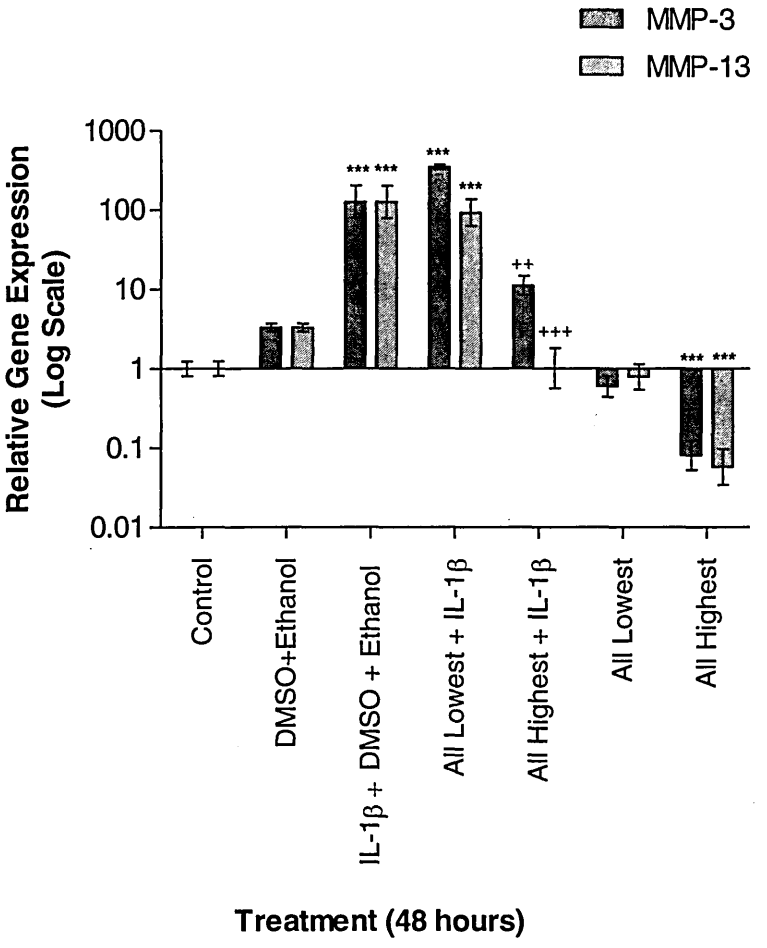

Figure 6.17 The effects of CB1, CB2 and PPARα, δ and γ agonists ACEA, HU308, Wy14643, GW0742, Troglitazone and Rosiglitazone on MMP-3 and -13 mRNA expression in OA chondrocytes cultured in monolayer. IL-1β stimulation induced MMP-3 and -13 mRNA expression. All agonists at a lower concentration of 2 μM, 1 μM, 10 μM, 0.2 μM, 1 μM and 10 μM for CB1 (ACEA), CB2 (HU308), PPARα (Wy14643), PPARδ (GW0742), PPARγ (troglitazone and rosiglitazone) respectively both alone and in combination with IL-1β had no effect on MMP-3 or -13 mRNA expression compared to DMSO control or IL-1β stimulation alone. All agonists at a higher concentration of 20 μM, 10 μM, 100 μM, 2 μM, 10 μM and 100 μM for CB1 (ACEA), CB2 (HU308), PPARα (Wy14643), PPARδ (GW0742), PPARγ (troglitazone and rosiglitazone) respectively both alone an in combination with IL-1β reduced the expression of both MMP-3 and -13 mRNA expression compared to DMSO control and IL-1β stimulation alone. Data represents mean fold change of mRNA expression normalised to internal reference gene and untreated control ± SEM.***$p<0.001$ compared to DMSO control and ++$p<0.01$, +++$p<0.001$ compared to IL-1β stimulation. n=6 obtained from two patient samples.

6.5.4 The Effects of Cannabinoid Receptor Agonists on ERK1/ERK2 Phosphorylation

6.5.4.1 The Effect of IL-1β on ERK1/ERK2 Phosphorylation

IL-1β stimulation for 30 minutes significantly induced ERK1/ERK2 phosphorylation compared to DMSO control ($p<0.001$) (Figure 6.18A-C, 6.19A-C).

6.5.4.2 The Effects of CB1, CB2 and PPAR Agonists on ERK1/ERK2 Phosphorylation

CB1 agonist (ACEA) and CB2 agonist (HU308) pre-treatment for 48 hours with the addition of PPARα (Wy14643), PPARδ (GW0742), PPARγ (troglitazone) or PPARγ (rosiglitazone) agonists in combination with IL-1β for the last 30 minutes significantly reduced ERK1/ERK2 phosphorylation compared to IL-1β stimulation alone for 30 minutes ($p<0.05$) and DMSO control ($p<0.05$) (Figure 6.18A). CB1 (ACEA) and CB2 (HU308) agonist treatment in combination for 48 hours reduced ERK1/ERK2 phosphorylation compared to DMSO control, however this was not significant (Figure 6.18A). CB1 (ACEA) and CB2 (HU308) agonist treatment in combination for 48 hours with the addition of PPARα (Wy14643), PPARδ (GW0742), PPARγ (troglitazone) or PPARγ (rosiglitazone) agonists reduced ERK1/ERK2 phosphorylation compared to DMSO control ($p<0.05$) (Figure 6.18A-C, 6.19A-C). Pre-treatment for 48 hours with all agonists combined at a lower concentration in combination with IL-1β for the last 30 minutes induced a significant increase in ERK1/ERK2 phosphorylation compared to DMSO control ($p<0.05$) (Figure 6.19C). Pre-treatment for 48 hours with all agonists at a higher concentration in combination with IL-1β significantly reduced the phosphorylation of ERK1/ERK2 compared to IL-1β stimulation alone and DMSO control ($p<0.05$) (Figure 6.19C). Following treatment with all agonists for 48 hours at a lower concentration there was no significant effect on ERK1/ERK2 phosphorylation compared to DMSO control (Figure 6.19C). Treatment with all agonists for 48 hours at a higher concentration resulted in a significant decrease in ERK1/ERK2 phosphorylation compared to DMSO control ($p<0.05$) (Figure 6.19C).

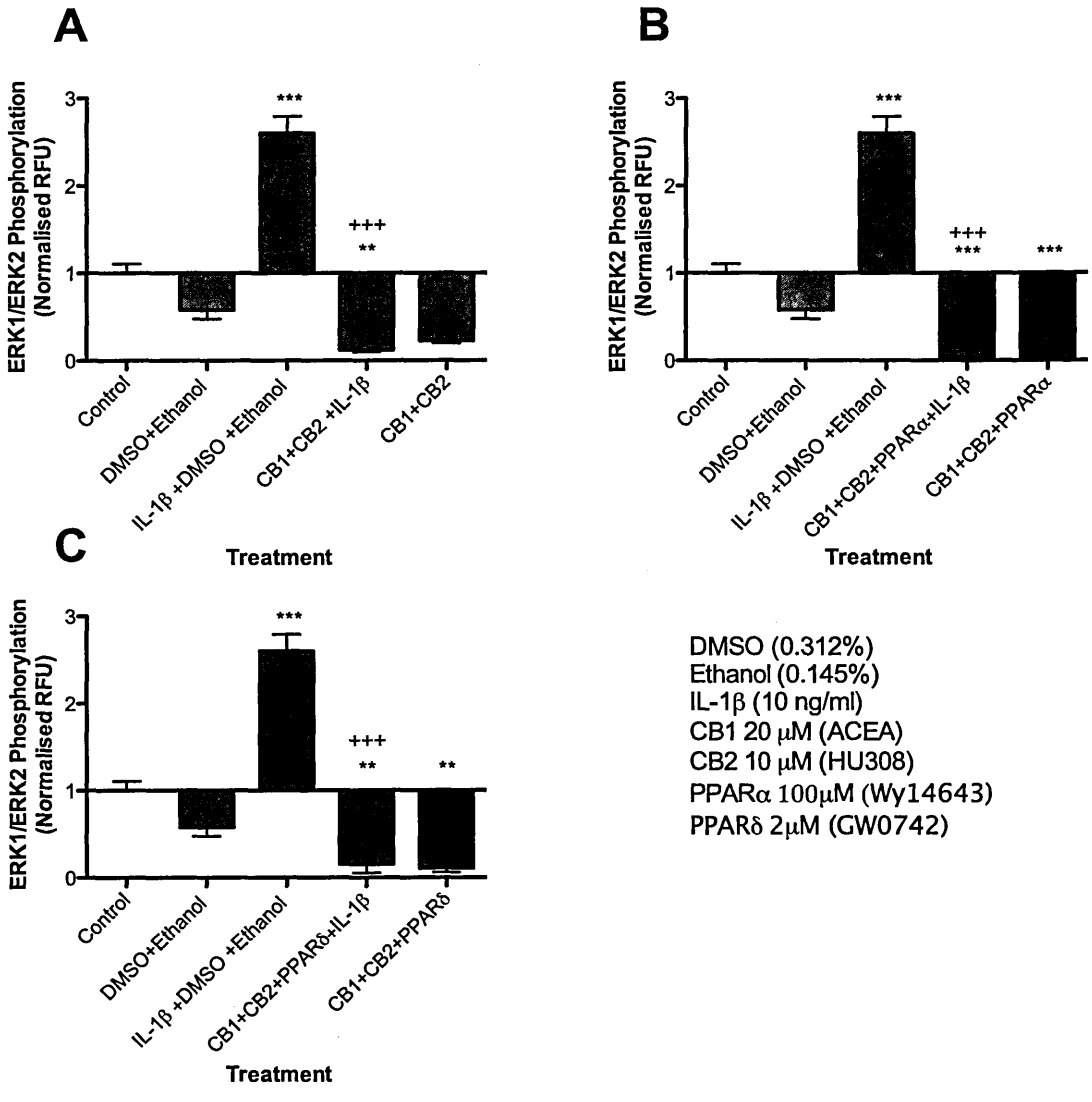

Figure 6.18 The effects of cannabinoid receptor agonists (A) CB1 (ACEA) and CB2 (HU308), (B) CB1 (ACEA) CB2 (HU308) and PPARα (Wy14643), (C) CB1 (ACEA) CB2 (HU308) and PPARδ (GW0742) on ERK1/ERK2 phosphorylation. IL-1β stimulation for 30 minutes induced the phosphorylation of ERK1/ERK2. CB1 (ACEA) and CB2 (HU308) pre-treatment for 48 hours with the addition of PPARα (Wy14643) or PPARδ (GW0742) in combination with IL-1β for the last 30 minutes reduced ERK1/ERK2 phosphorylation compared to basal and IL-1β levels. CB1 (ACEA) and CB2 (HU308) treatment for 48 hours with the addition of PPARα (Wy14643) or PPARδ (GW0742) reduced the basal levels of ERK1/ERK2 phosphorylation. Data represents mean fold change of phosphorylation normalised to internal total unphosphorylated protein and untreated control ± SEM. **p<0.01 ***p<0.001 compared to DMSO control, +++p<0.001 compared to IL-1β stimulation for 30 minutes.

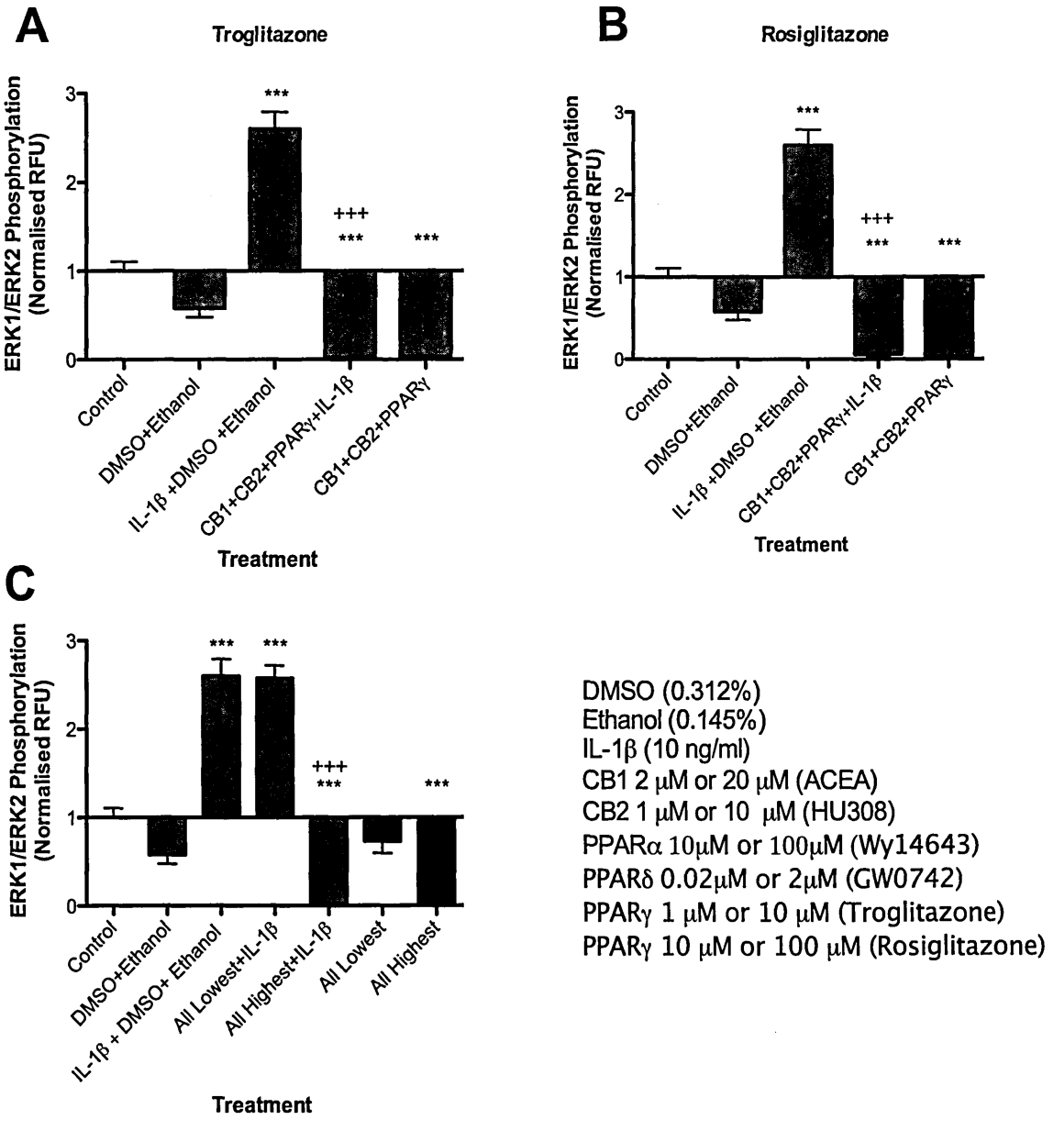

Figure 6.19 The effects of cannabinoid receptor agonists (A) CB1 (ACEA) and CB2 (HU308), (B) CB1 (ACEA) CB2 (HU308) and PPARγ (troglitazone), (C) CB1 (ACEA) CB2 (HU308) and PPARγ (rosiglitazone) on ERK1/ERK2 phosphorylation. IL-1β stimulation for 30 minutes induced the phosphorylation of ERK1/ERK2. CB1 (ACEA) and CB2 (HU308) pre-treatment for 48 hours with the addition of PPARγ (troglitazone or rosiglitazone) in combination on with IL-1β for the last 30 minutes reduced ERK1/ERK2 phosphorylation compared to basal and IL-1β levels. CB1 (ACEA) and CB2 (HU308) treatment for 48 hours with the addition of PPARγ (troglitazone or rosiglitazone) reduced the basal levels of ERK1/ERK2 phosphorylation. All agonists pre-treatment at a lower concentration in combination with IL-1β stimulation for the last 30 minutes had no effect on basal ERK1/ERK2 phosphorylation. All agonist pre-treatment at a higher concentration in combination with IL-1β stimulation for the last 30 minutes reduced ERK1/ERK2 compared to basal and IL-1β levels. All agonists at a lower concentration had no effect on ERK1/ERK2 phosphorylation. All agonists at a higher concentration treatment for 48 hours reduced ERK1/ERK2 phosphorylation below basal levels. Data represents mean fold change of phosphorylation normalised to internal total unphosphorylated protein and untreated control ± SEM. ***p<0.001 compared to DMSO control, +++p<0.001 compared to IL-1β stimulation for 30 minutes.

6.5.5 The Effects of Cannabinoid Receptor Agonists on c-Jun Phosphorylation

6.5.5.1 The Effect of IL-1β on c-Jun Phosphorylation

IL-1β stimulation for 30 minutes significantly induced c-Jun phosphorylation compared to DMSO control ($p<0.001$) (Figure 6.20A-D 6.21A & B).

6.5.5.2 The Effects of CB1, CB2 and PPAR Agonists on c-Jun Phosphorylation

CB1 (ACEA) and CB2 (HU308) agonist pre-treatment for 48 hours in combination with IL-1β for the last 30 minutes significantly reduced c-Jun phosphorylation compared to IL-1β stimulation alone ($p<0.01$), however phosphorylation remained significantly higher than DMSO control ($p<0.01$) (Figure 6.20 A). CB1 (ACEA) and CB2 (HU308) agonist treatment in combination for 48 hours significantly induced c-Jun phosphorylation compared to DMSO control ($p<0.01$) (Figure 6.20A). CB1 (ACEA), CB2 (HU308) and PPARα (Wy14643) agonist pre-treatment for 48 hours in combination with IL-1β stimulation for the last 30 minutes significantly reduced c-Jun phosphorylation compared to IL-1β alone ($p<0.001$) but not DMSO control (Figure 6.20B). CB1 (ACEA), CB2 (HU308) and PPARα (Wy14643) agonist treatment for 48 hours had no significant effect on c-Jun phosphorylation (Figure 6.20B). CB1 (ACEA), CB2 (HU308) and PPARδ (GW0742) agonist pre-treatment for 48 hours in combination with IL-1β stimulation for the last 30 minutes reduced c-Jun phosphorylation compared to IL-1β alone however statistical analysis could not be performed as phosphorylation was only detected in two of the repeats. CB1 (ACEA), CB2 (HU308) and PPARδ (GW0742) agonist treatment for 48 significantly induced c-Jun phosphorylation compared to DMSO control (Figure 6.20C).

CB1 (ACEA), CB2 (HU308) and PPARγ (troglitazone or rosiglitazone) agonist pre-treatment for 48 hours in combination with IL-1β stimulation for the last 30 minutes significantly reduced c-Jun phosphorylation compared to IL-1β alone ($p<0.01$, $p<0.001$), but not below basal levels (Figure 6.21A&B). CB1 (ACEA), CB2 (HU308) and PPARγ (troglitazone or rosiglitazone) agonist treatment for 48 hours had no significant effect on c-Jun phosphorylation compared to DMSO control (Figure 6.21A&B).

Pre-treatment for 48 hours with all agonists combined at a lower concentration in combination with IL-1β for the last 30 minutes induced a significant increase in c-Jun phosphorylation compared to DMSO control ($p<0.05$) (Figure 6.21C). Pre-treatment for 48 hours with all agonists at a higher concentration in combination with IL-1β for the last 30 minutes significantly reduced the phosphorylation of c-Jun compared to IL-1β stimulation alone and DMSO control ($p<0.05$) (Figure 6.21C). Following treatment with all agonists for 48 hours at a lower concentration there was no significant effect c-Jun phosphorylation compared to DMSO control (Figure 6.21C). Treatment with all agonists for 48 hours at a higher concentration slightly reduced c-Jun phosphorylation compared to DMSO control, however statistical analysis could not be performed as phosphorylation was only detected in one of the repeats (Figure 6.21C).

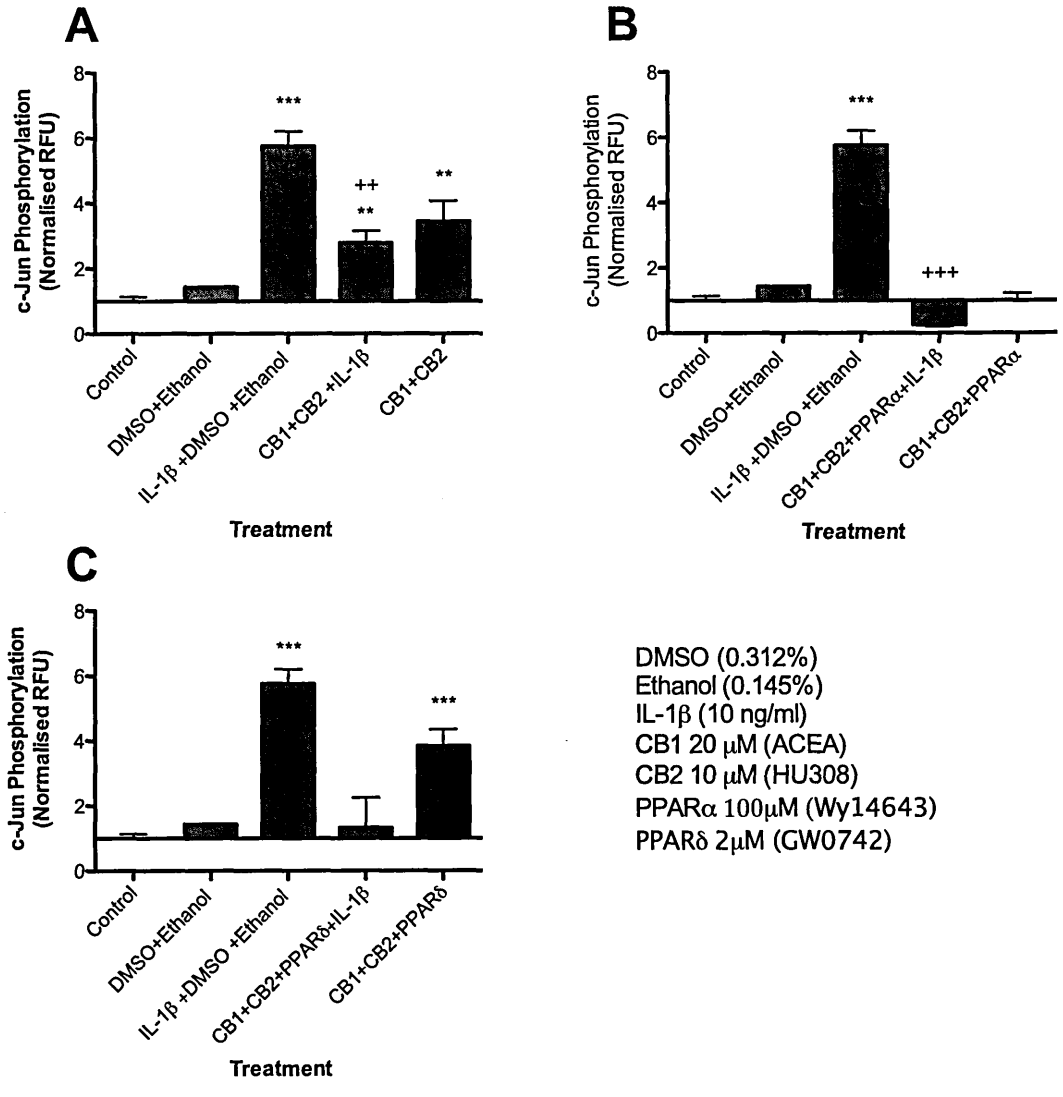

Figure 6.20 The effects of cannabinoid receptor agonists (A) CB1 (ACEA) and CB2 (HU308), (B) CB1 (ACEA) CB2 (HU308) and PPARα (Wy14643), (C) CB1 (ACEA) CB2 (HU308) and PPARδ (GW0742) on c-Jun phosphorylation. IL-1β stimulation for 30 minutes induced the phosphorylation of c-Jun. CB1 (ACEA) and CB2 (HU308) pre-treatment for 48 hours in combination with IL-1β for the last 30 minutes reduced c-Jun phosphorylation compared to IL-1β levels. CB1 (ACEA) and CB2 (HU308) treatment for 48 hours induced c-Jun phosphorylation. CB1 (ACEA) and CB2 (HU308) agonist pre-treatment for 48 hours with the addition or PPARα (Wy14643) or PPARδ (GW0742) agonists in combination with IL-1β for the last 30 minutes reduced c-Jun phosphorylation compared to IL-1β levels. CB1 (ACEA) and CB2 (HU308) agonist treatment for 48 hours with the addition or PPARα (Wy14643) or PPARδ (GW0742) agonists had no effect or induced c-Jun phosphorylation respectively. Data represents mean fold change of phosphorylation normalised to internal total unphosphorylated protein and untreated control ± SEM. **p<0.01, ***p<0.001 compared to DMSO control, ++p<0.01, +++p<0.001 compared to IL-1β stimulation for 30 minutes.

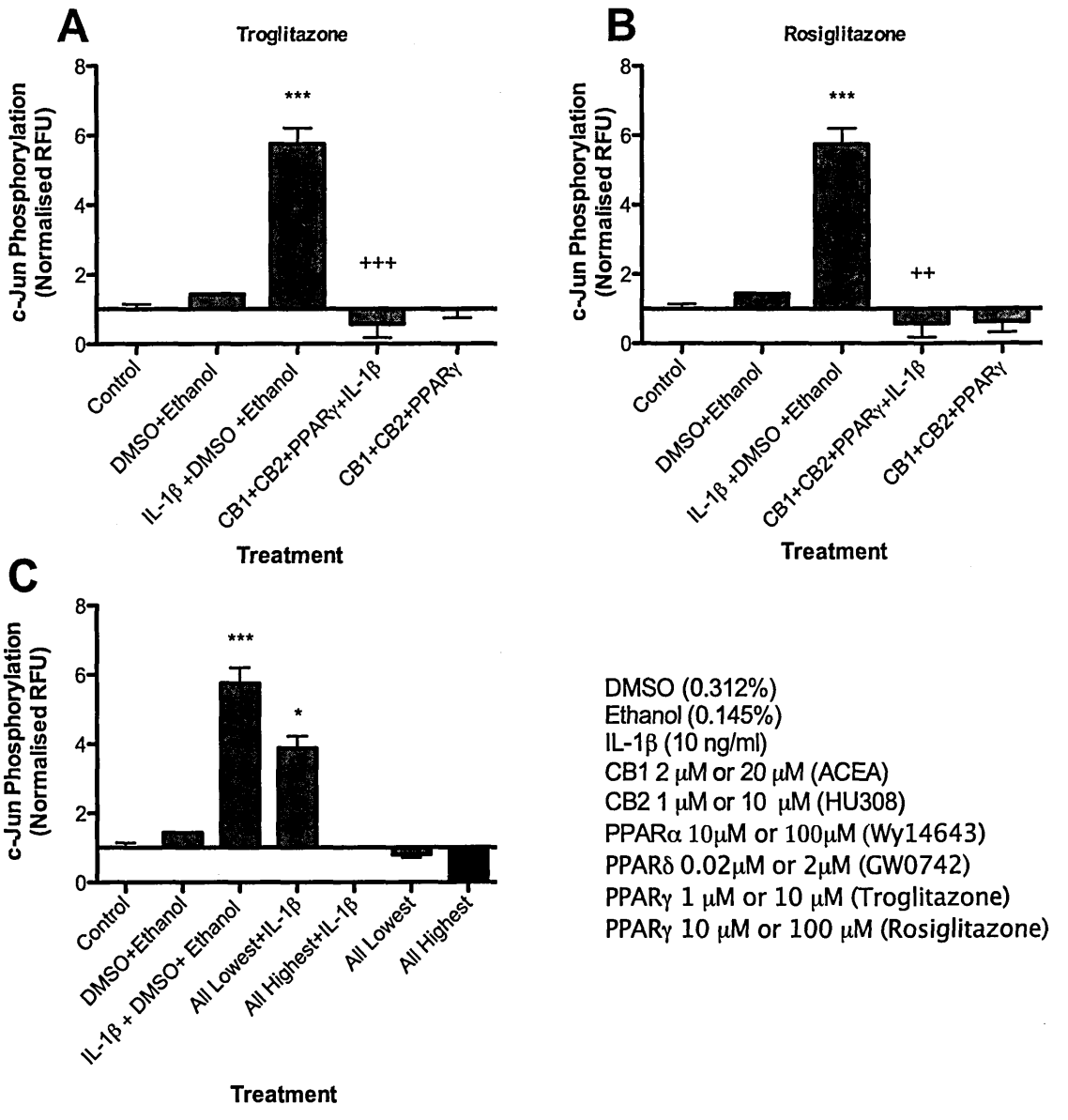

Figure 6.21 The effects of cannabinoid receptor agonists (A) CB1 (ACEA) and CB2 (HU308), (B) CB1 (ACEA) CB2 (HU308) and PPARγ (troglitazone), (C) CB1 (ACEA) CB2 (HU308) and PPARγ (rosiglitazone) on c-Jun phosphorylation. IL-1β stimulation for 30 minutes induced the phosphorylation of c-Jun. CB1 (ACEA) and CB2 (HU308) pre-treatment for 48 hours with the addition of PPARγ (troglitazone or rosiglitazone) in combination on with IL-1β for the last 30 minutes reduced c-Jun phosphorylation compared to IL-1β levels. CB1 (ACEA) and CB2 (HU308) treatment for 48 hours with the addition of PPARγ (troglitazone or rosiglitazone) had no effect on basal c-Jun. All agonists pre-treatment at a lower concentration in combination with IL-1β stimulation for the last 30 minutes induced c-Jun phosphorylation. All agonists pre-treatment at a higher concentration in combination with IL-1β stimulation for the last 30 minutes abolished c-Jun phosphorylation. All agonists at a lower concentration had no effect on basal c-Jun phosphorylation. All agonists at a higher concentration treatment for 48 hours reduced c-Jun phosphorylation below basal levels. Data represents mean fold change of phosphorylation normalised to internal total unphosphorylated protein and untreated control ± SEM. *$p<0.05$, ***$p<0.001$ compared to DMSO control, ++$p<0.01$, +++$p<0.001$ compared to IL-1β stimulation for 30 minutes.

6.5.6 The Effects of Cannabinoid Receptor Agonists on p38 Phosphorylation

6.5.6.1 The Effect of IL-1β on p38 Phosphorylation

IL-1β stimulation for 30 minutes significantly induced p38 phosphorylation compared to DMSO control ($p<0.01$) (Figure 6.22 A-D 6.23 A & B).

6.5.6.2 The Effects of CB1, CB2 and PPAR Agonists on p38 Phosphorylation

CB1 (ACEA) and CB2 (HU308) agonist pre-treatment for 48 hours in combination with IL-1β for the last 30 minutes significantly reduced p38 phosphorylation compared to IL-1β stimulation alone ($p<0.01$) (Figure 6.22A). p38 phosphorylation remained at basal levels, following CB1 (ACEA) and CB2 (HU308) agonist treatment both alone for 48 hours and in combination with IL-1β for the last 30 minutes (Figure 6.22A). CB1 (ACEA), CB2 (HU308) and PPARα (Wy14643) agonist pre-treatment for 48 hours in combination with IL-1β stimulation for the last 30 minutes reduced p38 phosphorylation compared to IL-1β alone and DMSO alone, however p38 phosphorylation was only detected in one of the repeats, therefore statistical analysis could not be performed (Figure 6.22B). CB1 (ACEA), CB2 (HU308) and PPARα (Wy14643) agonist treatment for 48 hours had no significant effect on p38 phosphorylation (Figure 6.20C). CB1 (ACEA), CB2 (HU308) and PPARδ (GW0742) agonist pre-treatment for 48 hours in combination with IL-1β stimulation for the last 30 minutes induced an increase in p38 phosphorylation compared to DMSO alone, however p38 phosphorylation was only detected in one of the repeats, therefore statistical analysis could not be performed (Figure 6.22C). CB1 (ACEA), CB2 (HU308) and PPARδ (GW0742) agonist treatment for 48 hours had no significant effect on p38 phosphorylation (Figure 6.22C). CB1 (ACEA), CB2 (HU308) and PPARγ (troglitazone) agonist pre-treatment for 48 hours in combination with IL-1β stimulation for the last 30 minutes decreased p38 phosphorylation compared to IL-1β alone and DMSO alone, however p38 phosphorylation was only detected in two of the repeats, therefore statistical analysis could not be performed (Figure 6.23A). CB1 (ACEA), CB2 (HU308) and PPARγ (troglitazone) agonist treatment for 48 hours abolished p38 phosphorylation (Figure 6.23A). CB1 (ACEA), CB2 (HU308) and PPARγ (rosiglitazone) agonist pre-treatment for 48

hours in combination with IL-1β stimulation for the last 30 minutes decreased p38 phosphorylation compared to IL-1β alone and DMSO alone, however p38 phosphorylation was only detected in one of the repeats, therefore statistical analysis could not be performed (Figure 6.23B). CB1 (ACEA), CB2 (HU308) and PPARγ (rosiglitazone) agonist treatment for 48 hours had no effect on p38 phosphorylation compared to DMSO control, however p38 phosphorylation was only detected in one of the repeats, therefore statistical analysis could not be performed (Figure 6.23B). Pre-treatment for 48 hours with all agonists combined at a lower concentration in combination with IL-1β for the last 30 minutes induced a significant increase in p38 phosphorylation compared to DMSO control ($p<0.01$) (Figure 6.23C). Following treatment with all agonists for 48 hours at a lower concentration there was no significant effect p38 phosphorylation compared to DMSO control (Figure 6.23C). Pre-treatment for 48 hours with all agonists at a higher concentration in combination with IL-1β for the last 30 minutes abolished p38 phosphorylation (Figure 6.23C). Treatment with all agonists for 48 hours at a higher concentration abolished p38 phosphorylation (Figure 6.23C).

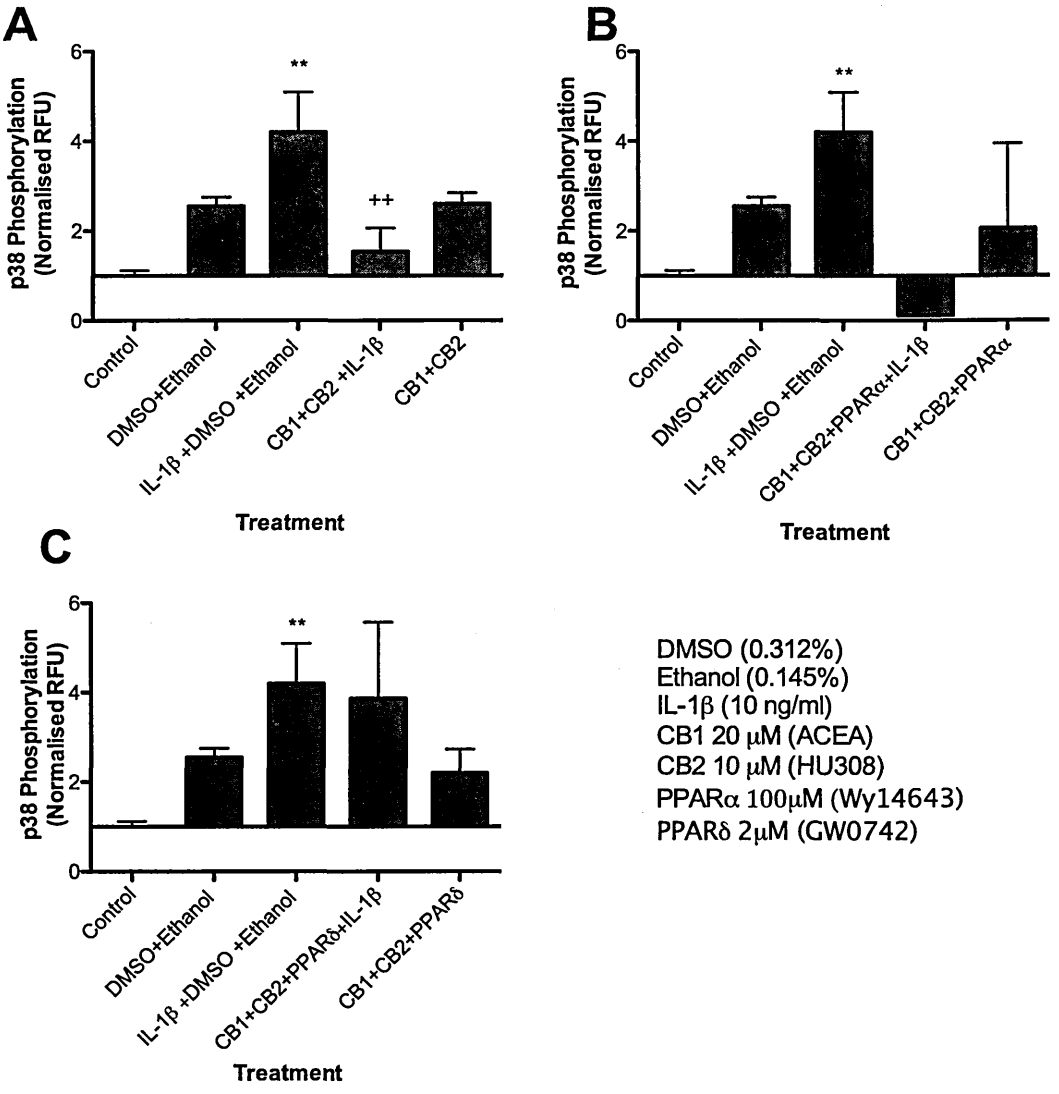

Figure 6.22 The effects of cannabinoid receptor agonists (A) CB1 (ACEA) and CB2 (HU308), (B) CB1 (ACEA) CB2 (HU308) and PPARα (Wy14643), (C) CB1 (ACEA) CB2 (HU308) and PPARδ (GW0742) on p38 phosphorylation. IL-1β stimulation for 30 minutes induced the phosphorylation of p38. CB1 (ACEA) and CB2 (HU308) pre-treatment for 48 hours in combination with IL-1β for the last 30 minutes reduced p38 phosphorylation compared to IL-1β levels. CB1 (ACEA) and CB2 (HU308) treatment for 48 hours had no effect on basal p38 phosphorylation. CB1 (ACEA) and CB2 (HU308) agonist pre-treatment for 48 hours with the addition or PPARα (Wy14643) agonist in combination with IL-1β for the last 30 minutes reduced p38 phosphorylation compared to IL-1β levels. CB1 (ACEA) and CB2 (HU308) agonist pre-treatment for 48 hours with the addition PPARδ (GW0742) agonist in combination with IL-1β for the last 30 minutes induced p38 phosphorylation. CB1 (ACEA) and CB2 (HU308) agonists treatment for 48 hours with the addition of PPARα (Wy14643) or PPARδ (GW0742) had no effect p38 phosphorylation. Data represents mean fold change of phosphorylation normalised to internal total unphosphorylated protein and untreated control ± SEM. **p<0.01 compared to DMSO control, ++p<0.01 compared to IL-1β stimulation for 30 minutes.

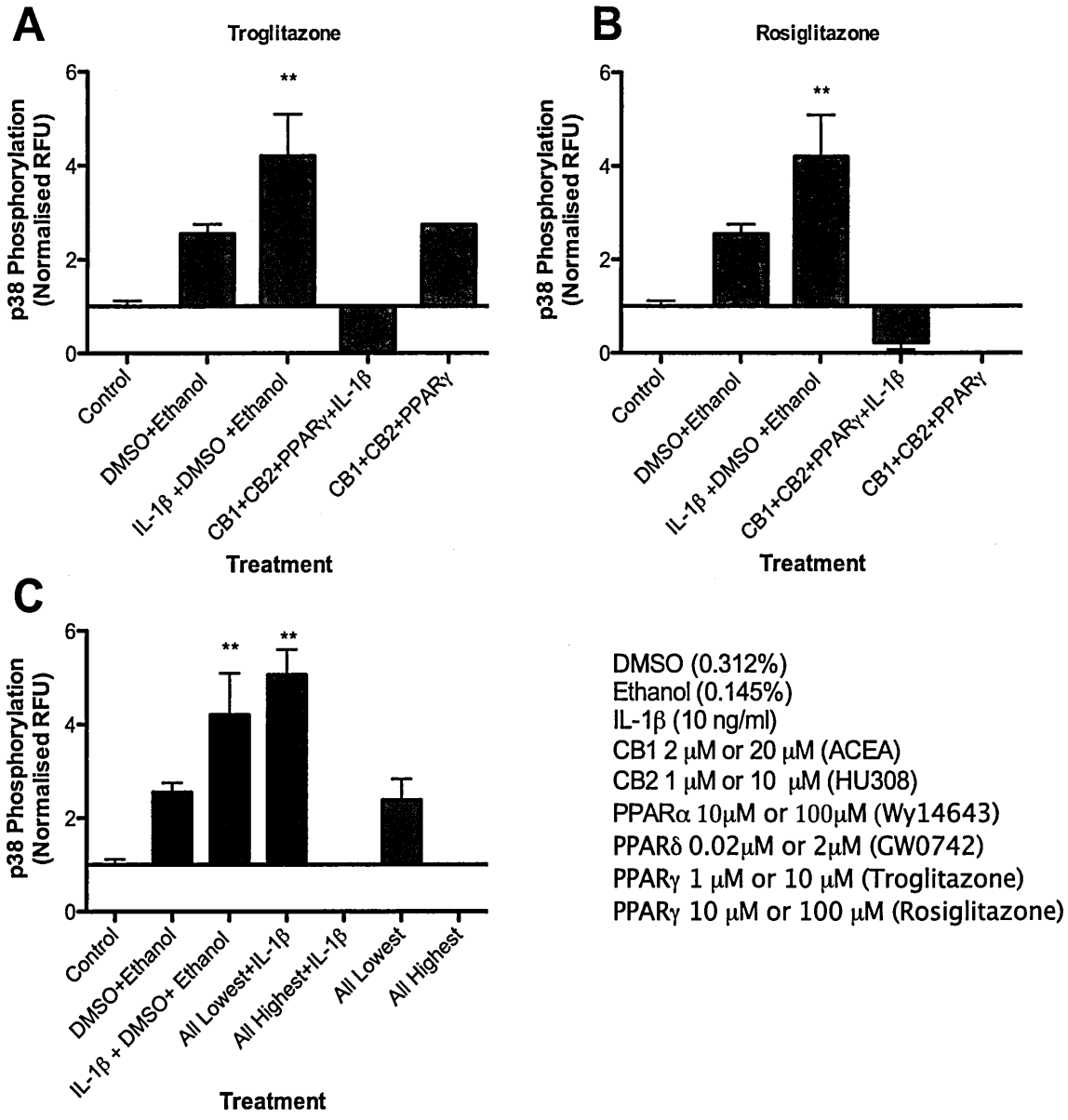

Figure 6.23 The effects of cannabinoid receptor agonists (A) CB1 (ACEA) and CB2 (HU308), (B) CB1 (ACEA) CB2 (HU308) and PPARγ (troglitazone), (C) CB1 (ACEA) CB2 (HU308) and PPARγ (rosiglitazone) on p38 phosphorylation. IL-1β stimulation for 30 minutes induced the phosphorylation of p38. CB1 (ACEA) and CB2 (HU308) pre-treatment for 48 hours with the addition of PPARγ (troglitazone or rosiglitazone) in combination with IL-1β for the last 30 minutes reduced p38 phosphorylation compared to IL-1β levels. CB1 (ACEA) and CB2 (HU308) treatment for 48 hours with the addition of PPARγ (troglitazone or rosiglitazone) had no effect or abolished p38 phosphorylation respectively. All agonists pre-treatment at a lower concentration in combination with IL-1β stimulation for the last 30 minutes induced p38 phosphorylation. All agonists pre-treatment at a higher concentration in combination with IL-1β stimulation for the last 30 minutes abolished p38 phosphorylation. All agonists at a lower concentration had no effect on basal p38 phosphorylation. All agonists at a higher concentration treatment for 48 hours abolished p38 phosphorylation. Data represents mean fold change of phosphorylation normalised to internal total unphosphorylated protein and untreated control ± SEM. **p<0.01 compared to DMSO control.

6.5.7 The Effects of Cannabinoid Receptor Agonists on IκB Phosphorylation

6.5.7.1 The Effect of IL-1β on IκB Phosphorylation

IL-1β stimulation for 30 minutes had no significant effect on IκB phosphorylation compared to DMSO control (Figure 6.24 A-D 6.25 A & B).

6.5.7.2 The Effects of CB1, CB2 and PPAR Agonists on IκB Phosphorylation

CB1 (ACEA) and (CB2) HU308 agonist pre-treatment for 48 hours in combination with IL-1β for the last 30 minutes reduced IκB phosphorylation, compared to IL-1β alone and DMSO alone, however statistical analysis could not be performed as phosphorylation was only detected in two of the repeats (Figure 6.24A). There was no significant effect on IκB phosphorylation following CB1 (ACEA) and CB2 (HU308) agonist treatment alone for 48 hours compared to DMSO control (Figure 6.24A). CB1 (ACEA), CB2 (HU308) and PPARα (Wy14643) agonist pre-treatment for 48 hours in combination with IL-1β for the last 30 minutes abolished IκB phosphorylation (Figure 6.24B). CB1 (ACEA), CB2 (HU308) and PPARα (Wy14643) agonist treatment for 48 hours reduced IκB phosphorylation below basal levels, however statistical analysis could not be performed as phosphorylation was only detected in two of the repeats (Figure 6.24B). CB1 (ACEA), CB2 (HU308) and PPARδ (GW0742) agonist pre-treatment for 48 hours in combination with IL-1β for the last 30 minutes significantly decreased IκB phosphorylation compared to IL-1β stimulation alone and DMSO control ($p<0.01$) (Figure 6.24C). CB1 (ACEA), CB2 (HU308) and PPARδ (GW0742) agonist treatment for 48 hours significantly reduced IκB phosphorylation compared to DMSO control ($p<0.01$) (Figure 6.24C). CB1 (ACEA), CB2 (HU308) and PPARγ (troglitazone) agonist pre-treatment for 48 hours in combination with IL-1β for the last 30 minutes decreased IκB phosphorylation compared to IL-1β stimulation alone and DMSO control, however IκB phosphorylation was only detected in two of the repeats, therefore statistical analysis could not be performed (Figure 6.25A). CB1 (ACEA), CB2 (HU308) and PPARγ (trogliazone) agonist treatment for 48 hours abolished IκB phosphorylation (Figure 6.25A). CB1 (ACEA), CB2 (HU308) and PPARγ (rosiglitazone) agonist pre-treatment for 48 hours in combination with IL-1β for the last 30 minutes significantly decreased IκB phosphorylation compared to IL-

1β stimulation alone ($p<0.01$) and DMSO control ($p<0.05$) (Figure 6.25B). CB1 (ACEA), CB2 (HU308) and PPARγ (rosiglitazone) agonist treatment decreased IκB phosphorylation, however IκB phosphorylation was only detected in one of the repeats, therefore statistical analysis could not be performed (Figure 6.25B). Pre-treatment for 48 hours with all agonists combined at a lower concentration in combination with IL-1β for the last 30 minutes had no significant effect on IκB phosphorylation compared to IL-1β stimulation alone and DMSO control (Figure 6.25C). Pre-treatment for 48 hours with all agonists at a higher concentration in combination with IL-1β for the last 30 minutes reduced IκB phosphorylation, however statistical analysis could not be performed as phosphorylation was only detected in one sample repeat (Figure 6.25C). Following treatment with all agonists for 48 hours at a lower concentration there was no significant effect on IκB phosphorylation compared to DMSO control (Figure 6.25C). Following treatment with all agonists for 48 hours at a higher concentration significantly reduced IκB phosphorylation compared to DMSO control ($p<0.05$) (Figure 6.25C).

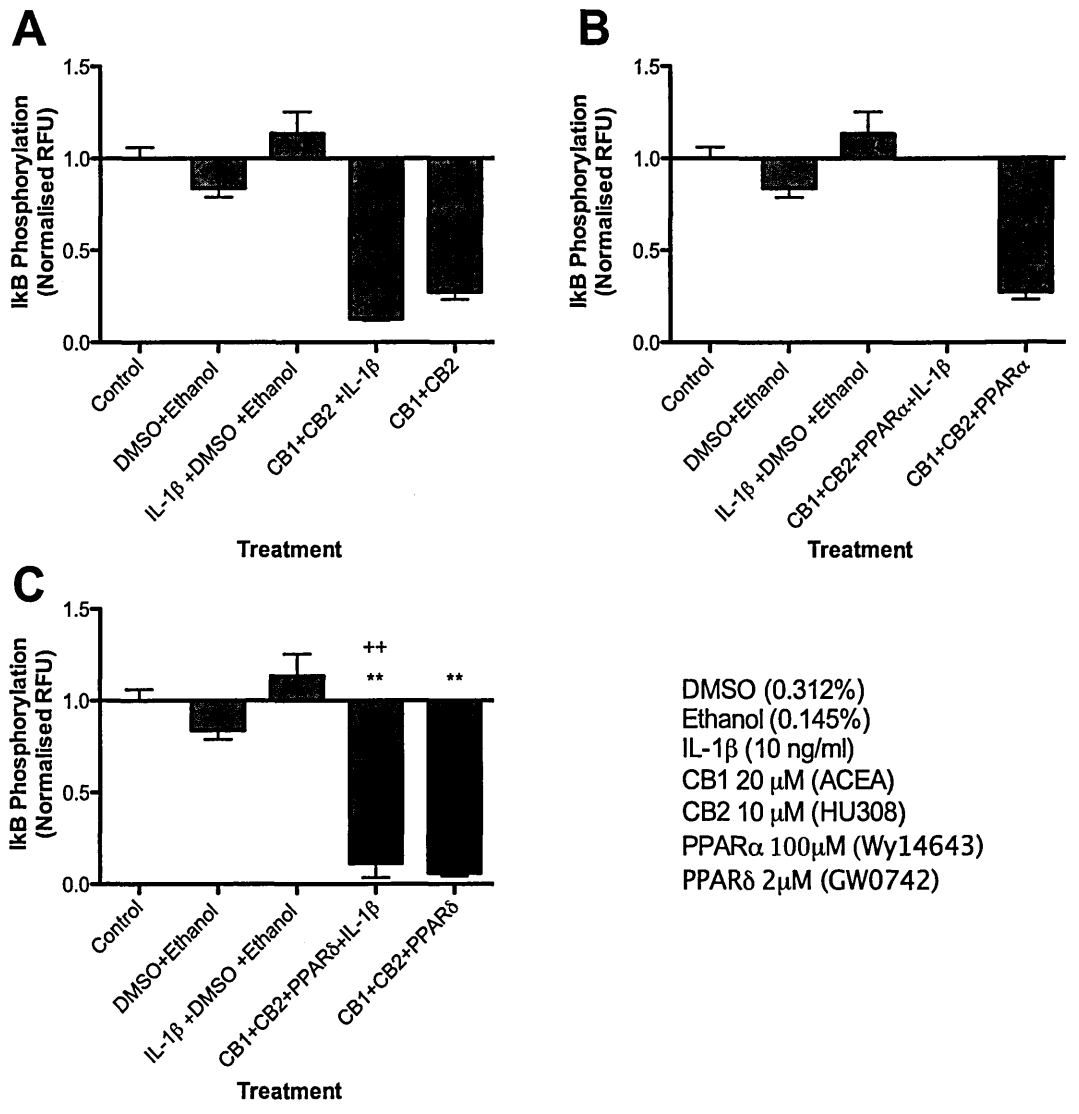

Figure 6.24 The effects of cannabinoid receptor agonists (A) CB1 (ACEA) and CB2 (HU308), (B) CB1 (ACEA) CB2 (HU308) and PPARα (Wy14643), (C) CB1 (ACEA) CB2 (HU308) and PPARδ (GW0742) on IκB phosphorylation. IL-1β stimulation for 30 minutes had no effect on IκB phosphorylation. CB1 (ACEA) and CB2 (HU308) pre-treatment for 48 hours alone or in combination with IL-1β for the last 30 minutes reduced IκB phosphorylation compared to basal or IL-1β levels. CB1 (ACEA) and CB2 (HU308) agonist pre-treatment for 48 hours with the addition or PPARα (Wy14643) or PPARδ (GW0742) agonists in combination with IL-1β for the last 30 minutes abolished or reduced IκB phosphorylation compared to IL-1β levels repectively. CB1 (ACEA) and CB2 (HU308) agonists treatment for 48 hours with the addition of PPARα (Wy14643) or PPARδ (GW0742) reduced basal IκB phosphorylation. Data represents mean fold change of phosphorylation normalised to internal total unphosphorylated protein and untreated control ± SEM. **p<0.01 compared to DMSO control, ++p<0.01 compared to IL-1β stimulation for 30 minutes.

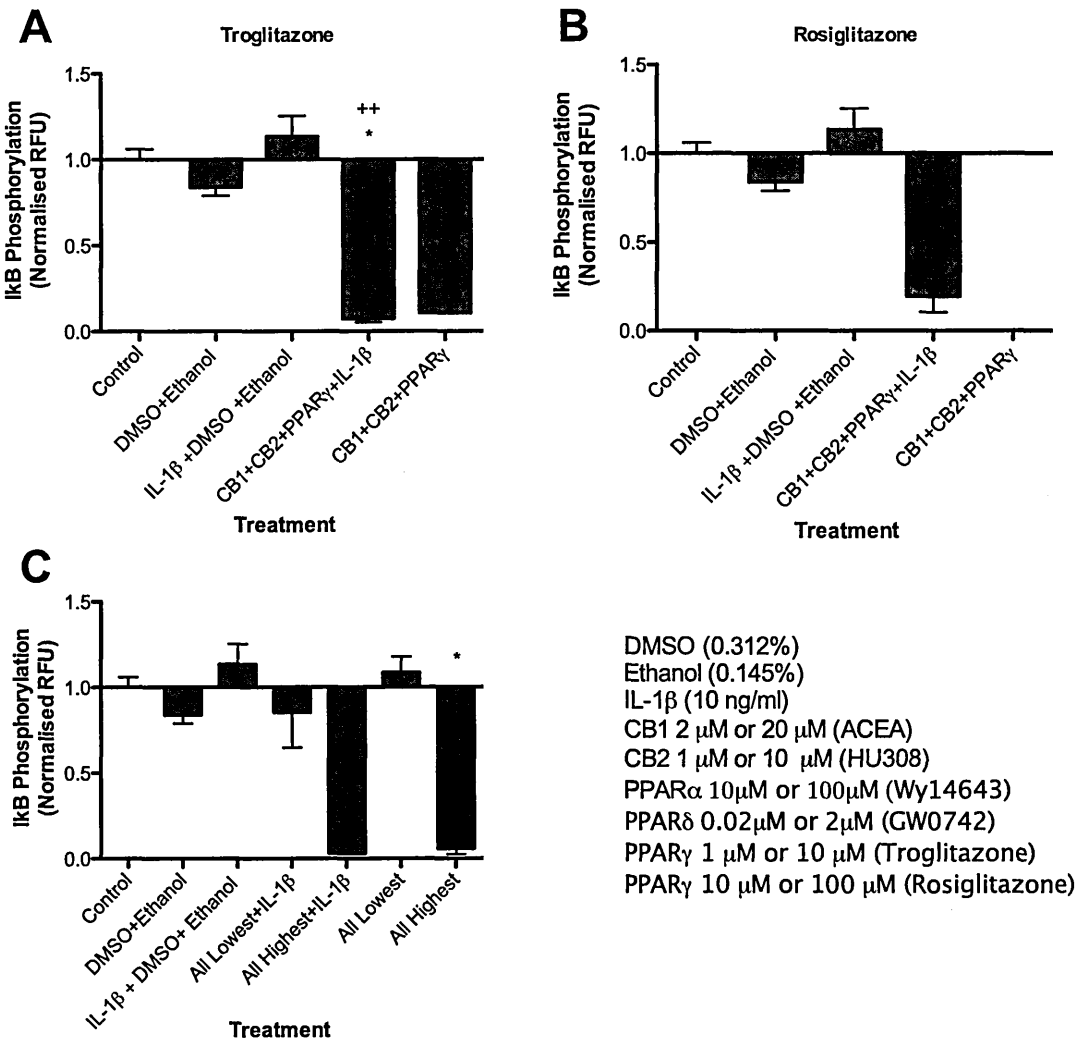

Figure 6.25 The effects of cannabinoid receptor agonists (A) CB1 (ACEA) and CB2 (HU308), (B) CB1 (ACEA) CB2 (HU308) and PPARγ (troglitazone), (C) CB1 (ACEA) CB2 (HU308) and PPARγ (rosiglitazone) on IκB phosphorylation. IL-1β stimulation for 30 minutes had no effect on IκB phosphorylation. CB1 (ACEA) and CB2 (HU308) pre-treatment for 48 hours with the addition of PPARγ (troglitazone or rosiglitazone) in combination on with IL-1β for the last 30 minutes reduced IκB phosphorylation compared to IL-1β levels. CB1 (ACEA) and CB2 (HU308) treatment for 48 hours with the addition of PPARγ (troglitazone or rosiglitazone) reduced or abolished IκB phosphorylation respectively. All agonists pre-treatment at a lower concentration both alone and in combination with IL-1β stimulation for the last 30 minutes had no effect on IκB phosphorylation. All agonists pre-treatment at a higher concentration, both alone and in combination with IL-1β stimulation for the last 30 minutes decreased IκB phosphorylation. Data represents mean fold change of phosphorylation normalised to internal total unphosphorylated protein and untreated control ± SEM. *$p<0.05$, compared to DMSO control, ++$p<0.01$ compared to IL-1β stimulation for 30 minutes.

6.5.8 Summary of Results

The summary of the findings presented in this chapter for the affects of cannabinoid receptor agonists on IL-1β induced MMP-3 and -13 mRNA expression and phosphorylation of signalling kinases analysis in OA chondrocyte cultures are shown in Tables 6.3 and 6.4.

Cannabinoid Receptor	Cannabinoid Receptor Agonist	MMP-3 mRNA expression compared to IL-1β treatment			MMP-13 mRNA expression compared to IL-1β treatment		
CB1	ACEA (μM)	0.2	2	20	0.2	2	20
		–	–	–	–	–	–
CB2	HU308 (μM)	0.1	1	10	0.1	1	10
		–	–	–	–	–	–
GPR55	LPI (μM)	0.1	1	10	0.1	1	10
		–	–	–	–	–	–
GPR18	NAGly (μM)	0.3	3	30	0.3	3	30
		–	–	–	–	–	–
TRPV1	OLDA (μM)	0.03	0.3	3	0.03	0.3	3
		–	–	–	–	↑+	↑++
PPARα	Wy14643 (μM)	1	10	100	1	10	100
		–	–	–	–	–	↑++
PPARδ	GW0742 (μM)	0.02	0.2	2	0.02	0.2	2
		–	–	–	–	–	–
PPARγ	Troglitazone (μM)	0.1	1	10	0.1	1	10
		–	–	–	–	–	–
PPARγ	Rosiglitazone (μM)	10		100	10		100
		–		–	–		–

Table 6.3 Summary of the effects of indivdual cannabinoid receptor agonists on IL-1β induced MMP-3 and -13 mRNA expression.

Cannabinoid Receptor	Cannabinoid Receptor Agonist (+10 ng/ml IL-1β)	MMP-3 mRNA expression compared to IL-1β treatment	MMP-13 mRNA expression compared to IL-1β treatment	ERK1/ERK2 compared to IL-1β treatment	c-Jun compared to IL-1β treatment	p38 compared to IL-1β treatment	IκB compared to IL-1β treatment
CB1 and CB2	ACEA (20 μM) and HU308 (10 μM)	–	–	↓***	↓**	↓**	↓
CB1, CB2 and PPARα	ACEA (20 μM), HU308 (10 μM) and Wy14643 (100 μM)	–	–	↓***	↓***	↓	❖
CB1, CB2 and PPARδ	ACEA (20 μM), HU308 (10 GW0742 (2 μM) and μM)	–	–	↓***	↓	–	↓**
CB1, CB2 and PPARγ	ACEA (20 μM), HU308 (10 μM) and troglitazone (10 μM)	–	–	↓***	↓**	↓	↓
CB1, CB2 and PPARγ	ACEA (20 μM), HU308 (10 μM) and rosiglitazone (100 μM)	–	–	↓***	↓***	↓	↓**
CB1, CB2 and WIN-55	ACEA (20 μM), HU308 (10 μM) and WIN-55 (10 μM)	↓***	↓***	Not investigated			
CB1, CB2 and PPARα, δ and γ	ACEA (2 μM), HU308 (1 μM), Wy14643 (10 μM), GW0742 (0.2 μM) troglitazone (1 μM) rosiglitazone (10 μM)	–	–	–	–	–	–
CB1, CB2 and PPARα, δ and γ	ACEA (20 μM), HU308 (10 μM), Wy14643 (10 μM), GW0742 (2 μM) troglitazone (10 μM) rosiglitazone (100 μM)	↓**	↓***	↓***	❖	❖	↓

Table 6.4 Summary of the effects of combinational cannabinoid receptor agonists treatments on IL-1β induced MMP-3 and -13 mRNA expression and signalling pathways. ❖Indicates phosphorylation was abolished.

6.6 Discussion

This study aimed to determine which cannabinoid receptor(s) shown to be expressed by OA chondrocytes (Chapter 5), mediate WIN-55 induced reduction of MMP-3 and -13 in the presence of IL-1β.

6.6.1 Individual Cannabinoid Receptor Activation

6.6.1.1 CB1 and CB2

In the present study, ACEA or HU308 treatments for 48 hours did not counteract the effects of IL-1β on induction of MMP-3 and -13 mRNA expression in OA chondrocytes. This may suggest that although having activity at CB1 and CB2 receptors (Pertwee *et al,* 2010). WIN-55 does not mediate its effects via these receptors alone in human OA chondrocytes. Interestingly in another study, WIN-55 was shown to inhibit IL-1β induced activation of NFκB and chemokine IL-8 expression in human astrocytes, however these effects were shown to be independent of CB1 and CB2 receptors as antagonists SR14176A and SR144528 or pertussis toxin had no effect on WIN-55 actions (Curran *et al,* 2005). Furthermore, in RA and OA fibroblast-like synoviocytes WIN-55 reduced IL-1β induced IL-6 and IL-8 secretion, similarly these effects were shown to be via a non CB1 or CB2 receptor mediated mechanism, as CB1 and CB2 antagonists AM281 and AM630 failed to affect the inhibitory actions of WIN-55 (Selvi *et al,* 2008). In addition, incubation of RA and OA fibroblast-like synoviocytes with CB1 agonist ACEA and CB2 agonist JWH-015 also had no effect on IL-1β induced IL-6 and IL-8 secretion (Selvi *et al,* 2008). In another study, using mouse macrophages, WIN-55 down regulated MMP-9 expression via reduction in ERK1/ERK2 signalling pathways. These effects were found to be independent of CB1 and CB2 receptors since AM51 and AM630 antagonist and pertussis toxin failed to inhibit WIN-55 induced effects (Tauber *et al,* 2012). Further, in bovine chondrocytes, CB1 and CB2 synthetic agonist HU210 along with WIN-55 were shown to inhibit IL-1α stimulated collagen and proteoglycan degradation, these effects were suggested to be mediated by CB1 and CB2, however, it is likely that these effects may also be mediated by other cannabinoid receptors expressed by chondrocytes since AM281 and AM630 (CB1 and CB2 cannabinoid receptor antagonists respectively) failed to counteract WIN-55 activities (Mbvundula *et al,* 2006).

6.6.1.2 GPR55

GPR55 has been identified as a possible cannabinoid receptor as it is activated by a number of cannabinoid receptor agonists (Ryberg *et al,* 2007). However there is conflicting data as to which cannabinoid ligands activate GPR55. Ryberg *et al* 2007 demonstrated using GTPγS binding assays that CB1 ligand CP55940, endogenous cannabinoid ligands AEA and 2-AG and phytocannabinoid THC bind to and activate GPR55. In addition phytocannabinoid ligands CBD and abnormal CBD, which display no activities at CB1 and CB2, were shown to act on GPR55 as an antagonist and agonist respectively (Ryberg *et al,* 2007). Laucke *et al* 2009 also demonstrated that THC and AEA activated GPR55, however in contrast 2-AG, CP55940, CBD and abnormal CBD were shown not to activate GPR55 as determined by intracellular calcium levels (Lauckner *et al,* 2008). Lastly, Kapur *et al* 2009 demonstrated, using the formation of β-arrestin-GPR55 complex and ERK1/ERK2 phosphorylation as indicators of GPR55 activation readouts that CB1 antagonists AM251 and SR141716A and the non-cannabinoid ligand LPI acted as GPR55 agonists. Moreover it was demonstrated that CP55940 acted as a GPR55 antagonist and partial agonist (Kapur *et al,* 2009). These conflicting findings may therefore be dependent on the assay system used to study the activation of GPR55.

In the present study it was shown that LPI had no effect on IL-1β induced MMP-3 or -13 mRNA expression, suggesting that WIN-55 does not mediate its effects via GPR55. In agreement with the findings presented here, Kapur *et al* (2009) demonstrated that WIN-55 displayed no activities at GPR55. Interestingly, abnormal CBD analogue O-1602 was shown to reduce inflammatory pain in a rat model of acute arthritis (Schuelert and McDougall 2011). These effects were thought to be mediated by GPR55, as GPR55 antagonist O-1918 blocked the effects of O-1602. Interestingly CB1 and CB2 antagonists AM281 and AM630 had no effect on O-1602 nociception activities, indicating that the effects were CB1 and CB2 independent (Schuelert and McDougall 2011).Together, these findings suggested activation of GPR55 although not reducing destructive pathways in OA, may modulate analgesic effects, however the significance of this remains to be determined.

6.6.1.3 GPR18

GPCR GPR18, although structurally different from the classical CB1 and CB2 receptors (Pertwee *et al,* 2010) is thought to play a role in endocannabinoid signalling in microglial-neuronal communication and migration in the CNS (McHugh 2012). In BV-2 microglia, HEK293-GRP18 and HEC-1B-GPR18 transfected cells, abnormal CBD and NAGly endogenous metabolite of AEA, induced cellular migration and MAPK signalling (McHugh *et al,* 2012; McHugh *et al,* 2010). Moreover, in siRNA GPR18 knockdown studies, NAGly induced cell migration in BV-2 cells was attenuated (McHugh *et al,* 2012) GPR18 is activated by other cannabinoid receptor agonists AEA and THC inducing migration and ERK1/ERK2 phosphorylation in HEC-1B cells, effects that were antagonised by pertussis toxin, AM251 and CBD (McHugh *et al,* 2012).Together these findings suggested that GPR18 plays a role in cannabinoid signalling independent of CB1 or CB2 receptors and GPR18 may be a therapeutic target in neurodegenerative diseases. However, in the present study NAGly failed to reduce IL-1β induced MMP-3 and -13 mRNA expression, suggesting WIN-55 does not mediate its effects via activation of GPR18.

6.6.1.4 TRPV1

In the present study it was shown that TRPV1 receptor agonist OLDA did not counteract the effects of IL-1β induced MMP-3 and -13 mRNA expression. In contrast, at 0.3 and 3 μM OLDA further increased IL-1β induced MMP-13 mRNA expression, however when used alone had no effect on basal levels of MMP-3 or -13. Interestingly, activation of TRPV1 with vanilloid agonist capsaicin induced the secretion of IL-6 from OA and RA synovial fibroblasts, these effects were attenuated by incubation with TRPV1 antagonist capsazepine (Engler *et al,* 2007). Conversely, cannabinoid agonist CBD induced anti-inflammatory effects in a rat model of acute inflammation, where the TPRV1 antagonist capsazeoine (CPZ) reversed the effects of CBD, whereas SR141716 and SR144528 CB1 and CB2 specific antagonists respectively had no effect (Costa *et al,* 2004). Together these findings suggest that activation of TRPV1 may have opposing effects depending on the agonists used. Interestingly, WIN-55 was shown to reduce IL-6 and IL-8 secretion from IL-1β stimulated OA and RA fibroblast-like synoviocytes via a non-TRPV1 mediated manner, since incubation with TRPV1 antagonist capsazepine failed to modify WIN-55 inhibitory effects on cytokine

production (Selvi *et al,* 2008). In an animal model of lung inflammation using monocyte-macrophages, WIN-55 was shown to inhibit MMP-9 secretion, effects that were thought to be mediated by TRPV1, since TRPV1 antagonist capsazpine also inhibited MMP-9 secretion (Tauber *et al,* 2012). In addition, WIN-55 actions were antagonised by TPRV1 agonist capsaicin (Tauber *et al,* 2012).

6.6.1.5 PPARα

In vivo pre-treatment with PPARα selective agonist Wy14643 inhibited LPI induced secretion of pro-inflammatory cytokines IL-6 and IFNγ and attenuated LPI induced infiltration of inflammatory cells in a mouse model of acute lung injury, these effects were not observed in PPARα knockout mice, suggesting that activation of PPARα displays anti-inflammatory properties (Yoo *et al,* 2013). In addition to anti-inflammatory properties activation of PPARα with Wy14643 was shown to have chondroprotective effects in OA (Clockaerts *et al,* 2011). In contrast, in the present study 1, 10 and 100 μM Wy14643 failed to counteract IL-1β induced MMP-3 or -13 mRNA expression in monolayer cultures of human OA chondrocytes, and when used at 100 μM in combination with IL-1β further increased MMP-13 mRNA expression compared to IL-1β stimulation alone. In other studies, in OA cartilage explants, 100 μM Wy14643 decreased IL-1β induced mRNA expression of MMP-1, -3 and -13 and secretion of NO and PGE_2 and release of GAGs, whilst having no effect on the expression of collagen type II or aggrecan (Clockaerts *et al,* 2011). Of note when used at a lower concentration of 10 μM, Wy14643 had no effect on MMPs, GAGs, NO or PGE_2 (Clockaerts *et al,* 2011). In another study, using rabbit articular chondrocytes, PPARα agonist clofibrate, counteracted the IL-1β induced mRNA expression of MMP-1, -3 and -13 and induced the expression of IL-1Ra (Francois *et al,* 2006). To fully elucidate the effects of PPARα activation on catabolic pathways in OA requires further investigation.

6.6.1.6 PPARδ

There is little known regarding the effects of PPARδ activation in OA. In the present study, activation of PPARδ using the selective agonist GW0742 at a concentration of 0.02, 0.2 or 2 μM respectively, failed to counteract the effects of IL-1β on induction of MMP-3 and -13 mRNA expression in human OA chondrocytes. In rat synovial fibroblasts, rosiglitazone, although being a

selective PPARγ agonist, stimulated production of IL-1Ra via a PPARδ dependent mechanism, as the rosiglitazone induced expression of IL-Ra was abolished by transfection with a dominant negative form of PPARδ (Moulin *et al,* 2005). *In vivo,* GW0742 was shown to have anti-inflammatory effects in a mouse model of acute lung injury via the reduction of iNOS, TNFα, neutrophil infiltration and IκB-α degradation (Di Paola *et al,* 2010). Recently, activation of PPARδ with GW0742 in human chondrocytes stimulated with IL-1, decreased NO and IL-6 production (Paukkeri *et al,* 2013). In support of the findings present here GW0742 had no affect on MMP-3 production (Paukkeri *et al,* 2013). These findings suggest that PPARδ activation may have potential anti-arthritic properties via the upregulation of IL-1Ra and the modulation of anti-inflammatory mediators; however this remains to be determined in human OA.

6.6.1.7 PPARγ

PPARγ is known to play a role in modulating catabolic factors in OA and targeting this receptor with specific ligands has been investigated (Fahmi *et al,* 2011). In the present study, PPARγ agonists troglitazone and rosiglitazone failed to counteract IL-1β induced expression of MMP-3 and -13 in human OA chondrocytes. In contrast previous studies have shown that low concentrations of rosiglitazone (0.1-1μM) reduced IL-1β induced MMP-1, -3 and -13 expression and GAG release in rabbit articular chondrocytes, in a PPARγ dependent manner (Francois *et al,* 2004). Furthermore, it was demonstrated that the MMP-1 promoter contained a PPARγ binding site and that the inhibitory effect of rosiglitazone on IL-1β induced MMP-1 gene expression was via the binding of PPARγ (Francois *et al,* 2004). In RA fibroblast-like synovial cells, 1 or 10 μM of troglitazone inhibited the production of TNFα, IL-6, IL-8 and MMP-3 and supressed TNFα or IL-1β induced NFκB activation (Yamasaki *et al,* 2002). In contrast, 15d-PGJ_2 an endogenous ligand for PPARγ but not troglitazone decreased or abolished IL-1β induced mRNA expression of COX-2 and iNOS and release of prostaglandins and NO in human chondrocytes (Boyault *et al,* 2001). *In vivo,* PPARγ ligand pioglitazone reduced the development of cartilage lesions in a dog model of OA via the reduction of MMP-1, ADAMTS-5 and iNOS (Boileau *et al,* 2007). Pioglitazone also reduced the severity of OA in a guinea pig model via the reduction of MMP-13 and IL-1β (Kobayashi *et al,* 2005). Findings presented in this study are in contrast to previous studies and

demonstrate that activation of PPARγ does not inhibit IL-1β induced MMP-3 or -13 mRNA expression, therefore PPARγ receptors alone may not mediate the actions of WIN-55 in human OA chondrocytes. Interestingly, WIN-55 was also shown to mediate anti-inflammatory effects in OA and RA fibroblast-like synoviocytes via a PPARγ independent mechanism, since incubation with PPARγ antagonist GW9662 failed to mediate WIN-55 inhibitory effects on IL-1β induced IL-6 and IL-8 secretion (Selvi *et al,* 2008). Similarly, WIN-55 was shown to reduce MMP-9 expression in mouse macrophages in a PPARγ independent manner as antagonist GW9662, had not effect on WIN-55 actions (Tauber *et al,* 2012), suggesting that WIN-55 does not mediate inhibition of MMP-3 and -13 mRNA expression via an individual cannabinoid receptor expressed by OA chondrocytes. Other studies have supported the suggestion that WIN-55 mediates its effects via a novel cannabinoid receptor in joint cells. Selvi *et al* (2008) demonstrated that WIN-55 inhibitory effects on IL-8 and IL-6 secretion from OA and RA synovial fibroblasts were independent of CB1, CB2, PPARγ or TRPV1 receptors. Although in the present study activation of a single cannabinoid receptor using selective agonists had no effect on IL-1β induced MMP-3 or -13 mRNA, it may also be proposed that WIN-55 activates multiple cannabinoid receptors, which are co-expressed in OA chondrocytes (Chapter 5).

6.6.2 Cannabinoid Receptor Agonists Combination Effects.

Since individual activation of CB1, CB2 or PPAR α, δ and γ failed to reduce IL-1β induced MMP-3 and -13 expression in the present study, it is possible that WIN-55 may first activate CB1 and CB2 receptors expressed on the cell membrane which then leads to the activation of the nuclear receptors PPAR α, δ or γ individually or in combination (O'Sullivan and Kendall 2010) in order to induce its inhibitory effects on MMP-3 and MMP-13 expression and IL-1β signalling pathways. Combination treatments of selective agonists for CB1 and CB2 receptors were used in the present study to target cannabinoid receptors that are expressed on the cell membrane of OA chondrocytes (Chapter 5) and are receptors WIN-55 is know to activate (Pertwee *et al,* 2010). The addition of nuclear receptor agonists for PPAR α, δ and γ used individually or in combination with CB1 and CB2 receptors agonists were used as WIN-55 has been shown to activate PPARα and γ (O'Sullivan and Kendall 2010; Sun *et al,* 2006). WIN-55 also induced mRNA expression of PPARδ in human OA

chondrocytes (Chapter 3, section 5.5.3.2). Furthermore, it was shown that these cannabinoid receptors co-exist in OA chondrocytes (Chapter 5), suggesting these receptors are available for WIN-55 binding and activation.

Preliminary studies here have shown that treatment of human OA chondrocytes with CB1 and CB2 agonists ACEA and HU308 in combination or with the addition of either PPARα, δ and γ agonists Wy14643, GW0742, troglitazone or rosiglitazone respectively failed to counteract the effects of IL-1β induced MMP-3 or -13 mRNA expression, suggesting that WIN-55 does not mediate its effects via CB1 or CB2 receptors or one of the PPAR receptors alone. Interestingly, although having no effect on MMP-3 or -13 expression, pre-treatment of OA chondrocytes with CB1 and CB2 receptor agonists ACEA and HU308 for 48 hours counteracted the effects of IL-1β induced phosphorylation of ERK1/ERK2, c-Jun, p38 and IκB and treatment with ACEA and HU308 in combination reduced ERK1/ERK2 and IκB phosphorylation below basal levels, whilst having no effect on p38 phosphorylation. Interestingly ACEA and HU308 treatment induced c-Jun phosphorylation above basal levels. Previously, activation of CB1 with phytocannabinoid THC induced the phosphorylation of c-Jun as shown in CB1 transfected CHO cells, however, phosphorylation occurred within 30 minutes of stimulation (Rueda *et al,* 2000) which in contrast to the present study was shown following 48 hours of treatment. Cannabinoid agonists have been shown to signal through MAPK activation upon binding to their respective cannabinoid receptors (Howlett 2005; Demuth and Molleman 2006). Furthermore, in human synovial fibroblast-like cells obtained from patients with OA and RA, HU210 a CB1 and CB2 receptor agonist induced a time-dependent phosphorylation of ERK1/ERK2 and p38 following up to 10 minutes stimulation. Effects were reversed by the addition of pertussis toxin suggesting these effects were mediated by CB1 and CB2 receptors (Richardson *et al,* 2008). Phosphorylation of ERK1/ERK2 and p38 was used to identify functional CB1 and CB2 receptors in synovial fibroblast-like cells, however only a short incubation time was investigated (Richardson *et al,* 2008). In contrast in the present study, CB1 and CB2 receptor agonists reduced ERK1/ERK2 and p38 signalling, however, since in the present study 48 hours incubation time was investigated it is possible that phosphorylation occurs rapidly or signalling

through G-protein coupled receptors may produce distinctive effects in different types of cells (Luttrell and Luttrell 2003).

In this study, pre-treatment of OA chondrocytes with CB1, CB2 and PPARα receptor agonists ACEA, HU308 and Wy14643 repectively for 48 hours counteracted the effects of IL-1β induced phosphorylation of ERK1/ERK2, c-Jun, p38 and abolished IκB phosphorylation, furthermore treatment alone with ACEA, HU308 and Wy14643 reduced ERK1/ERK2 phosphorylation and IκB phosphorylation below basal levels. The chondroprotective activities of PPARα activation are in part thought to be mediated by the inhibition of NFκB by increasing the expression of IκB (Crisafulli and Cuzzocrea 2009). Furthermore PPARα agonist clofibrate decrease LPS stimulated phosphorylation of ERK, JNK and p38 in peritoneal mice macrophages, effects that were not seen in the absence of functional PPARα gene (Crisafulli and Cuzzocrea 2009). Interestingly ACEA and HU308 in combination with Wy14643 reduced IL-1β induced c-Jun phosphorylation, however ACEA and HU308 used in combination without Wy14643 induced c-Jun phosphorylation, these findings suggest that Wy14643 may counteract the effects of ACEA and HU308 induced c-Jun phosphorylation. Previous studies have shown that activation of PPARα reduces c-Jun and c-fos phosphorylation (Li *et al,* 2009). In addition, in the present study Wy14643 used in combination with ACEA and HU308 reduced IκB phosphorylation. In other studies, treatment with Wy14643 alone inhibited IL-1β induced translocation of NFκB to the nucleus in monolayer-cultured chondrocytes (Clockaerts *et al,* 2011). In the present study, whether the effects observed were mediated by activation of CB1, CB2 or PPARα individually or in combination remains to be determined.

Pre-treatment of OA chondrocytes with CB1, CB2 and PPARδ receptor agonists ACEA, HU308 and GW0742 for 48 hours counteracted the effects of IL-1β induced phosphorylation of ERK1/ERK2, c-Jun and IκB phosphorylation, whilst having no effect on p38 phosphorylation. Furthermore treatment alone with ACEA, HU308 and GW0742 reduced ERK1/ERK2 phosphorylation and IκB phosphorylation below basal levels, whilst having no effect on p38 phosphorylation, but increased c-Jun phosphorylation. However, whether these effects were mediated by activation of CB1, CB2 or PPARδ individually or in combination remains to be determined. Interestingly activation of PPARδ alone

using selective agonist GW501516, induced the phosphorylation of p38 and JNK in hepatic stellate cells and GW0742 reduced ERK1/ERK2 phosphorylation in a human keratinocyte cell line (Kostadinova *et al,* 2011; Burdick *et al,* 2007). Although PPARδ may mediate its effects via MAPK signalling, there is little knowledge as to the effects of PPARδ mediated MAPK signalling in OA chondrocytes. Furthermore, whether the effects demonstrated in the present study were mediated by activation of CB1, CB2 or PPARδ individually or in combination remains to be investigated.

Here, preliminary studies have shown that pre-treatment of OA chondrocytes with CB1, CB2 and PPARγ receptor agonists ACEA, HU308 and troglitazone for 48 hours counteracted the effects of IL-1β induced phosphorylation of ERK1/ERK2, c-Jun, p38 and IκB phosphorylation, furthermore treatment alone with ACEA, HU308 and troglitazone reduced ERK1/ERK2 phosphorylation below basal levels, abolished p38 and IκB phosphorylation whilst having no effect on the basal levels of c-Jun. Pre-treatment of OA chondrocytes with CB1, CB2 and PPARγ receptor agonists ACEA, HU308 and rosiglitazone for 48 hours counteracted the effects of IL-1β induced phosphorylation of ERK1/ERK2, c-Jun, p38 and IκB phosphorylation. Furthermore treatment alone with ACEA, HU308 and rosiglitazone reduced ERK1/ERK2 phosphorylation and IκB phosphorylation below basal levels, whilst having no effect on the basal levels of c-Jun or p38 phosphorylation. Together these findings suggest that activation of cannabinoid receptors with agonists reduces the phosphorylation of protein kinases involved in IL-1β signalling pathways whilst having no effect on MMP-3 or -13 mRNA expression. *In vivo,* using a dog model of OA, PPARγ agonist pioglitazone inhibited ERK1/ERK2, p38 and NFκB phosphorylation, although in contrast to the present study, where no effect on MMP-3 or MMP-13 mRNA expression was observed, a reduction of MAPK signalling resulted in a decrease in MMP-1 expression (Boileau *et al,* 2007). In the present study, CB1 and CB2 agonists ACEA and HU308 induced c-Jun phosphorylation when treated in combination, however the addition of rosiglitazone, troglitazone or Wy14643 but not GW0742, counteracted these effects, suggesting that alone PPARγ or α but not δ can reduce the phosphorylation of c-Jun.

Fahmi *et al* 2002 demonstrated that activation of PPARγ with rosiglitazone or 15d-PGJ_2 in human synovial fibroblasts reduced IL-1β induced expression of

MMP-1, furthermore 15d-PGJ_2 reduced IL-1β induced binding of AP-1 to the promoter region of MMP-1 (Fahmi *et al,* 2002). In human chondrocytes, rosiglitazone was shown to inhibit MMP-1 expression via PPARγ by the induction of DNA binding competition on a composite PPRE/AP-1 site within the MMP-1 promoter (Francois *et al* 2004). Since c-Jun phosphorylation is needed for the formation of the AP-1 complex it is possible that rosiglitazone in this study may also reduce AP-1 formation, although the significance of this needs to be investigated further, since both MMP-3 and -13 contain AP-1 binding sites (Mengshol *et al,* 2000; Vincenti 2001; Borden and Heller 1997; Mengshol *et al,* 2001; Vincenti and Brinckerhoff 2002; Goldring *et al,* 2011) and in the current study rosiglitazone failed to reduce IL-1β induced MMP-3 or MMP-13 mRNA expression. Interestingly, in rabbit articular chondrocytes rosiglitazone treatment alone was shown to have no effect on IL-1β induced NFκB activity following 18 hours treatment (Francois *et al,* 2004). In the present study, rosiglitazone used in combination with CB1 and CB2 receptor agonists ACEA and HU308 respectively, reduced IκB phosphorylation below basal levels, however whether these effects were induced by CB1, CB2 or PPARγ activation, remains to be determined.

All agonists for CB1, CB2 and PPARα, δ and γ including ACEA, HU308, Wy14643, GW0742 and troglitazone and rosiglitazone when used at a lower concentration failed to counteract the effects of IL-1β induced MMP-3 and -13 mRNA expression and ERK1/ERK2, c-Jun and p38 phosphorylation. However, when used at a 10 fold higher concentration all agonists combined reduced IL-1β induced MMP-3 and -13 mRNA expression. Other studies have shown that activation of PPARα or PPARγ alone using selective agonists reduced the expression of MMP-1, -3 and -13 in human OA cartilage and synovial fibroblasts (Fahmi *et al,* 2002; Clockaerts *et al,* 2011). In contrast here it was shown that activation of multiple cannabinoid receptors is required to reduce MMP-3 and -13 mRNA expression in human OA chondrocytes. Furthermore, in order for lipophilic cannabinoids, to activate PPARs, they need to pass through the cell membrane and the hydrophilic cytosol; the mechanism via which this occurs is unclear (O'Sullivan and Kendall 2010). However it has recently been suggested that fatty acid binding proteins FABP5 and FABP7 act as intracellular transporters for endogenous cannabinoid anandamide (Kaczocha *et al,* 2009),

suggesting that a similar mechanism for the transport of cannabinoids to PPARs may be present. Since WIN-55 is know to activate CB1, CB2 and PPARα and γ and data presented in Chapter 4 demonstrated that cannabinoid receptors co-exist in OA cartilage, it is possible that WIN-55 may mediate its effects via a number of receptors, firstly activating CB1 and CB2 cell surface receptors and initiating a cascade of intracellular signalling pathways resulting in the indirect activation of PPARs (O'Sullivan and Kendall 2010).

WIN-55 was shown to increase the expression of PPARα and δ (section 5.5.3.1 and 5.5.3.2). Interestingly, PPARγ has been shown directly interact with c-Jun preventing the formation of AP-1 complex thus having transcriptional suppressive effects on target genes such as MMPs (Vincenti and Brinckerhoff 2002; Delerive *et al,* 1999). In human chondrocytes, gel mobility and supershift assays demonstrated that PPARγ and c-fos/c-Jun proteins bind to the *cis*-acting element a composite of PPRE/AP1 site in the MMP-1 promoter thus preventing IL-1β induced expression (Francois *et al* 2004). Furthermore transactivation studies using c-Jun and c-fos plasmids have shown that activation of PPARα with Wy-14643 and PPARγ with troglitazone and rosiglitazone agonists interfere negatively with AP-1 transcription activity (Delerive *et al,* 1999). Therefore it is possible that an increase in PPARα and δ mRNA expression by WIN-55 is a prerequisite for the indirect inhibition of c-Jun phosphorylation via PPAR interaction. This is also supported by the findings that a 48 hour pre-treatment of WIN-55 is required for inhibition of IL-1β induced c-Jun phosphorylation (section 4.4.3.3) suggesting that an increase in PPAR mRNA expression induced by WIN-55 occurring following 48 hours (section 5.5.3.1 and 5.5.3.2). may be required to inhibit IL-1β induced c-Jun phosphorylation and activation which was shown to occur within 30 minutes of chondrocyte stimulation (section 4.4.3.3).

Together these findings suggest that WIN-55 may activate the classical cannabinoid receptors CB1 and CB2 expressed on the cell membrane followed by the direct or indirect activation of intracellular PPAR cannabinoid receptors. However, in order to determine fully the effects of cannabinoid receptor activation on MMP expression and MAPK signalling would require additional analysis to be performed on further patient samples. In addition, the effects of individual receptor agonists on ERK1/ERK2, c-Jun, p38 and IκB would need to

be investigated to determine if a combination of cannabinoid receptors or individual cannabinoid receptors mediate the reduction of MAPK and IκB signalling demonstrated in the present study. Furthermore, the effects of receptor agonists on the level of unphosphorylated total levels of MAPKs and IκB remain to be determined. The findings presented in this study need to be interpreted with caution since at high concentrations of cannabinoid agonists used in combination induced a significant reduction in cell viability.

6.6.3 Summary

WIN-55 is known to activate CB1, CB2 and PPARα and γ receptors (Pertwee *et al,* 2010; O'Sullivan 2007; Sun *et al,* 2006). The data presented here suggests that WIN-55 may activate a number of cannabinoid receptors including CB1, CB2 and all three subtypes of PPARs to inhibit IL-1β induced MMP-3 and -13 mRNA expression. However, at present this work does not identify which receptors need to be activated by WIN-55, directly or indirectly to produce the inhibition of MMP expression observed (Chapter 2, Dunn *et al,* 2013). Further investigation is therefore needed to identify specifically which receptors are involved in WIN-55 mediated effects in human OA chondrocytes. This works also suggests that WIN-55 may be mediating its effects via a yet unidentified novel receptor a possibility also reported in other studies (Selvi *et al,* 2008; Curran *et al,* 2005).

7 General Discussion and Future Directions

A key pathological feature of OA is cartilage degradation. The pro-inflammatory cytokine IL-1β plays a major role in pathogenesis of OA via the upregulation of matrix degrading enzymes principally MMP-1, -3 and -13 (Lefebvre *et al,* 1990; Mengshol *et al,* 2000; Reboul *et al,* 1996). IL-1β is synthesised by chondrocytes during OA and acts in an autocrine and paracrine manner binding to IL-1R1 expressed by chondrocytes inducing a cascade of intracellular signalling pathways, including MAPKs and NFκB (Attur *et al,* 1998; Martel-Pelletier *et al,* 1992; Vincenti and Brinckerhoff 2002). These pathways lead to the activation and translocation of transcription factors to the nucleus of stimulated cells inducing the expression of MMPs (Vincenti and Brinckerhoff 2002). In addition to inducing catabolic processes during OA, IL-1β also decreases the expression of anabolic genes including aggrecan and collagen II further contributing to cartilage loss (Goldring *et al,* 1994; Chadjichristos *et al,* 2003; Stove *et al,* 2000). Therefore, IL-1β signalling inhibition is thought to be an important target in the treatment of OA to prevent cartilage degradation (Kapoor *et al,* 2011).

Previous studies have shown that cannabinoids have protective activities in animal models of arthritis. Non-psychoactive synthetic cannabinoid AJA, reduced the severity of adjuvant-induced arthritis in addition to displaying anti-inflammatory properties (Zurier *et al,* 1998). Non-psychoactive phytocannabinoid CBD blocked the progression of collagen-induced arthritis and displayed anti-inflammatory properties via the reduction of IFN-γ and TNFα (Malfait *et al,* 2000) and HU-320 a metabolite of a synthetic homologue of cannabidiol reduced joint damage in collagen-induced arthritis (Sumariwalla *et al,* 2004).

The overall aim of this study was to determine the effects of synthetic cannabinoid WIN-55 on chondrocyte catabolic pathways and to investigate a possible mechanism via which its effects are mediated. The present study has shown a possible mechanism by which WIN-55 may act to prevent cartilage breakdown during OA via the inhibition of IL-1β induced MMP-3 and -13 at both the mRNA and protein level in a time and concentration dependent manner (Chapter 2). Interestingly, WIN-55 also decreased the expression of MMP inhibitors TIMP-1 and -2 in a time and concentration dependent manner suggesting that inhibition occurred via a signalling pathway common to both MMPs and TIMPs.

Cannabinoids have been shown to have anti-inflammatory effects *in vitro* (Mbvundula *et al,* 2004; Croxford and Yamamura 2005; Klein 2005). Furthermore studies have demonstrated that cannabinoids display analgesic activities in animal models of arthritis and cannabis based medicine Sativex has analgesic effects in patients with RA (Blake *et al,* 2006; Schuelert and McDougall 2011; Smith *et al,* 1998; Cox and Welch 2004; Cox *et al,* 2007; Schuelert and McDougall 2008). Therefore, the effects of WIN-55 on other OA targets including pro-inflammatory chemokine IL-8, the pain related peptide substance P and the neurotrophin NGF were investigated in human OA chondrocytes (Chapter 3).

IL-8 has been shown to play a role in cartilage degradation during OA via the upregulation of MMP-13 as shown in human and bovine chondrocytes and IL-8 has been shown to be increased in human OA chondrocytes and the synovial fluid of OA patients (Kaneko *et al,* 2000; Merz *et al,* 2003; Attur *et al,* 1998). Furthermore, IL-1β has been shown to induce the expression of IL-8 in human chondrocytes and IL-8 is thought to be involved in the inflammatory process of OA by the recruitment of neutrophils to the joint (Lotz *et al,* 1992; Elford and Cooper 1991). The present study has demonstrated that WIN-55 may also display anti-inflammatory effects in human OA chondrocytes as shown by the reduction of IL-1β induced IL-8 mRNA expression (Chapter 3). In agreement with findings presented here, previous studies have shown that WIN-55 inhibited IL-1β induced IL-8 secretion in human OA and RA synovial fibroblasts (Selvi *et al,* 2008).

Both NGF and substance P have been associated with pain in OA and the subchondral junction is the site of innervation where angiogenesis has been associated with the expression of NGF (Walsh *et al,* 2010; Keeble and Brain 2004). WIN-55 has been shown to have analgesic activities and reduce nociception in animal models of inflammatory pain (Ebrahimzadeh and Haghparast 2011; Burgos *et al,* 2010). Conversely, in the present study WIN-55 was shown to induce mRNA expression of neuropeptides NGF and substance P (Chapter 3). Although, substance P and NGF are known to be involved in pain signalling there is evidence to suggest differential roles for these factors in chondrocytes. NGF may possess anti-inflammatory actions as blocking of endogenous NGF in animal models induced joint inflammation (Manni *et al,*

2002). Moreover, NGF may have protective properties in human OA chondrocytes and has been suggested to stimulate chondrocytes metabolism and promote cartilage repair (Iannone *et al,* 2002). Other studies have shown that substance P may be involved in chondrocyte proliferation and cell adhesion contacts (Iannone and Lapadula 1998; Opolka *et al,* 2012). However, in contrast studies have shown a negative role of substance P in human OA chondrocytes via the up-regulation of MMP-13 (Im *et al,* 2008).

The effects of WIN-55 on IL-1β induced signalling pathways including MAPKs and NFκB were investigated to elucidate a possible mechanism via which WIN-55 inhibits MMPs (Chapter 4). WIN-55 was shown to inhibit IL-1β induced ERK1/ERK1, c-Jun and IκB but not p38. These findings suggest that WIN-55 inhibits distinct MAPK phosphorylation in human OA chondrocytes. Since both MMPs and TIMPs share a common AP-1 binding site in their promoters, it may be suggested that inhibition of IL-1β induced c-Jun phosphorylation demonstrated by WIN-55 in the present study reduces the formation of the AP-1 complex thus reducing the activation of both MMP and TIMP genes (Vincenti and Brinckerhoff 2002; Borden and Heller 1997). Further, data presented here suggests a possible mechanism via which WIN-55 may act to prevent IL-1β induced IL-8 expression via inhibition of its transcriptional regulation by NFκB and AP-1. Studies have shown that IL-8 has binding sites in its promoter for NFκB and AP-1 (Mukaida *et al,* 1994; Kunsch and Rosen 1993; Roebuck 1999). The present study has shown that WIN-55 decreased IL-1β induced IκB and c-Jun phosphorylation (Chapter 4), therefore potentially preventing the translocation of NFκB to the nucleus to induce target genes and the the activity of AP-1 transcription factor (Karin *et al,* 1997).

Cannabinoids were thought to mediate their effects via CB1 and CB2 receptors (Pertwee *et al,* 2010; Matsuda *et al,* 1990; Munro *et al,* 1993). It is now apparent that not all actions of cannabinoids are mediated via these receptors. Endogenous cannabinoid AEA and phytocannabinoid CBD are also known to act via TRPV1 and other GPCRs including GRP55 and GPR18 have been shown to bind a number of cannabinoid ligands (Figure 1.8) (McHugh *et al,* 2012; Bisogno *et al,* 2001; Smart and Jerman 2000; Kapur *et al,* 2009). In addition nuclear receptors PPARα and γ are known to be activated by cannabinoids (O'Sullivan 2007). In the present study it was shown that

cannabinoid receptors CB1, CB2, GPR55, GPR18, TRPV1 and PPARα, δ and γ are co-expressed in human OA articular cartilage and osteocytes (Chapter 5). Furthermore, cannabinoid receptors have been previously shown to be expressed by a number of joint cells including, chondrocytes, synoviocytes and bone cells (Table 1.3). In the present study the expression of putative cannabinoid receptors was investigated to determine if receptor expression was modulated in relation to grade of degeneration in different cartilage zones.

Immunohistochemical studies presented here showed that PPARγ expression was significantly decreased in osteocytes with increasing grade of cartilage degeneration. Furthermore, PPARγ was predominantly expressed in the superficial zone of the cartilage and a trend towards decrease in the chondrocytes present in proliferating clusters was observed although this did not reach significance. The importance of PPARγ in normal cartilage homeostasis has recently been described; *in vivo* studies in PPARγ cartilage specific knockout mice developed a spontaneous OA phenotype, as shown by an increase in cartilage degradation, hypocellularity, synovial inflammation, increase in MMP-13 and increased staining of MMP generated aggrecan and collagen type II neo-epitopes VDIPEN and C1-2C respectively (Vasheghani *et al,* 2013). Collectively these findings suggest PPARγ plays a role in ECM turnover under physiological conditions and a decrease in this receptor may be involved in the pathogenesis of OA.

GPR18 expression was increased in the middle and deep zone of the cartilage compared to the superficial zone, these findings suggest that GPR18 may display differential roles within different zones of the cartilage. There is evidence to suggest that chondrocytes display zonal differences as shown by *in vitro* studies in which chondrocytes isolated from the superficial zone and deep zone express different molecules such as lubricin and PTHrP expressed by superficial zone chondrocytes and Indian hedgehog and Runx-2 expressed by deep zone chondrocytes (Cheng *et al,* 2007; Eleswarapu *et al,* 2007; Chen *et al,* 2008). In addition TRPV1 was reduced in the deep zone of the cartilage with increasing grade of degeneration and previous studies have shown a decrease in TRPV1 expression to be associated with a differentiated phenotype in human OA chondrocytes cultures (Gavenis *et al,* 2009).

This study has demonstrated that WIN-55 treatment of human OA chondrocyte cultures induced the re-localisation/trafficking of cannabinoid receptors, including, CB1, CB2, GRP18, TRPV1 and PPARα and δ as shown by nuclear immunopositivity compared to cytoplasmic staining in control cells. These findings may indicate the rapid desensitisation and internalisation of GPCRs following agonist binding, principally CB1 and CB2 (Abood 2005). In addition, WIN-55 treatment is known to lead to the dephosphorylation of TRPV1, which has been shown to occur via the internalisation of the receptor (Jeske *et al,* 2006; Sanz-Salvador *et al,* 2012). Furthermore, PPARα has been shown to shuttle between the cytoplasm and nucleus following ligand activation (Umemoto and Fujiki 2012). These findings suggest that a process of cellular redistribution may regulate or contribute to the effects of cannabinoid receptor mediated chondroprotection.

There is increasing evidence to show activation of PPARs may modulate the anti-inflammatory response by inhibiting inflammatory mediator production and catabolic factors (O'Sullivan and Kendall 2010; Fahmi *et al,* 2001; Fahmi *et al,* 2002; Johnson *et al,* 2007; Clockaerts *et al,* 2011; Fahmi *et al,* 2011; Giaginis *et al,* 2009), suggesting that PPARs may be important in the development of new treatments for OA. The present study has demonstrated that WIN-55 significantly increased the mRNA expression of both PPARα and δ in human OA chondrocytes (Chapter 5), suggesting that WIN-55 may further reduce the expression of catabolic and inflammatory mediators in chondrocytes via the upregulation of these receptors.

The present study has shown that activation of individual cannabinoid receptors using selective agonists for CB1, CB2, GPR55, GPR18, TRPV1 and PPARα, δ and γ failed to counteract IL-1β induction of MMP-3 and -13 (Chapter 6), suggesting that WIN-55 does not mediate its effects via activation of an individual cannabinoid receptor. However, activation of cannabinoid receptors CB1, CB2 and PPARα, δ and γ using selective agonists in combination resulted in a significant decrease in IL-1β induced MMP-3 and -13 mRNA expression in human OA chondrocytes (Chapter 6). Preliminary investigations also demonstrated that a combination of cannabinoid receptor agonists reduced MAPKs and IκB signalling pathways (Chapter 6). WIN-55 is known to activate

CB1 and CB2 receptors in addition to PPARα and γ and the present study has demonstrated that WIN-55 induces the mRNA expression of PPARδ (Pertwee *et al,* 2010;O'Sullivan 2007;Sun *et al,* 2006). These findings suggest that WIN-55 may activate multiple cannabinoid receptors expressed by chondrocytes first on the cell membrane including CB1 and CB2 then directly or indirectly via the activation of PPARs expressed on the nuclear membrane (Figure 7.1).

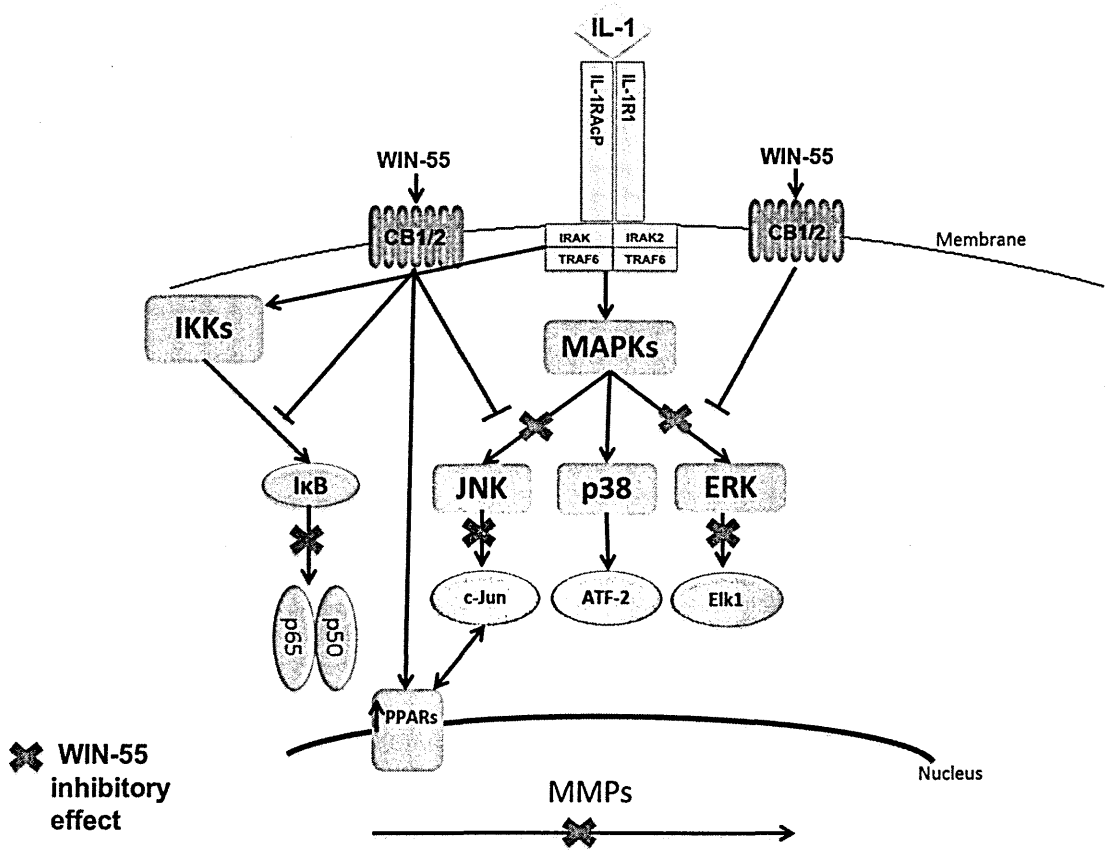

Figure 7.1 The potential effects of WIN-55 on IL-1β induced signaling pathways in human OA chondrocytes. WIN-55 binds to and activates the classical cannabinoid receptors CB1 and CB2 expressed on the cell membrane inhibiting the phosphorylation of MAPK signalling pathway JNKs and ERKs and IκB preventing the translocation of transcription factors to the nucleus thus preventing the expression of MMPs. WIN-55 may also induce the increased mRNA expression of PPARs principally PPARα, δ increasing PPAR/c-jun interaction thus having transcriptional suppressive effects on target genes such as MMPs.

Although WIN-55 is known to activate both CB1 and CB2 receptors with similar affinity (Pertwee *et al,* 2010) other studies have suggested that WIN-55 may mediate its effects via a mechanism(s) independent of the classical CB1 and CB2 receptors. Previous studies have shown that WIN-55 reduced IL-6 and IL-8 secretion from human OA and RA synovial fibroblasts; however CB1, CB2, PPARγ and TRPV1 antagonists failed to counteract WIN-55 effects suggesting WIN-55 mediates its effects independently of these receptors (Selvi *et al,* 2008). Furthermore in human astrocytes WIN-55 was shown to inhibit IL-1β signalling pathways in a CB1 and CB2 independent manner (Curran *et al,* 2005). Collectively, these findings suggest that WIN-55 may mediate its effects via a yet unidentified cannabinoid receptor or a receptor independent mechanism.

WIN-55 induces increases in intracellular calcium via CB1 activation, however these findings have been shown to be independent of $G_{i/o}$ coupling suggesting that WIN-55 activates CB1 via a novel mechanism. It has been proposed that WIN-55 stabilises the conformation of CB1, which couples to $G_q/11$ (Lauckner *et al,* 2005). WIN-55 is also thought to release calcium from intracellular stores via $G_q/11$ in contrast to the classical $G_{i/o}$ coupling (Lauckner *et al,* 2008). These findings indicate that WIN-55 mediates differential activation of CB1 and the intracellular signalling pathways induced by cannabinoid receptor activation are dependent on the agonist which binds and the conformation of G-protein coupling which is induced.

Recent studies have identified a possible mechanism via which cannabinoids namely AEA, are transported intracellularly via fatty acid binding protein, FABP5 and FABP7 (Kaczocha *et al,* 2009). This may provide a possible mechanism via which WIN-55 is transported to PPARs expressed on the nuclear membrane directly activating them. However, further investigation is required to establish how WIN-55 activates PPARs. In addition WIN-55 may indirectly activate PPARs. In the present study WIN-55 was shown to inhibit IL-1β induced c-Jun phosphorylation, which is required for the formation of the AP-1 complex, a key regulator of MMP gene expression. Interestingly, PPARγ is known to physically interact with c-Jun (Delerive *et al,* 1999), therefore it may be suggested that WIN-55 induces an AP-1/PPARγ association which is transcriptionally

repressive, thus decreasing the expression of MMP-3 and -13 (Vincenti and Brinckerhoff 2002). Furthermore transactivation studies have shown that activation of PPARα with Wy-14643 and PPARγ with troglitazone and rosiglitazone agonists interferes negatively with AP-1 transcription activity (Delerive *et al,* 1999). Since WIN-55 induces the mRNA expression of PPARα and δ (Chapter 5), this may be a possible mechanism via which MMP gene expression is decreased in human OA chondrocytes. Furthermore, PPARγ agonists have been shown to reduce IL-1β induced MMP-1 expression in human synovial fibroblasts and chondrocytes via inhibiting DNA binding of AP-1 (Fahmi *et al,* 2002).

7.1 Future Directions

Previously, broad range MMP inhibitors trialled for the treatment of OA have failed due to toxicity issues inhibition of MMP-1 is thought to contribute to skeletal deformities and lack of selectivity (Murphy and Nagase 2008; Krzeski *et al,* 2007; Tu *et al,* 2008). The present study has shown that WIN-55 inhibits both MMP-3 and -13 in a time and concentration dependent manner at the mRNA level. Since this study has only focused on inhibition of MMP-3 and -13 it would be interesting to determine the effects of WIN-55 on other MMPs which are thought to contribute to OA pathogenesis (Table 1.2) using a broader approach to analyse the expression of multiple MMPs with the use of mRNA and protein arrays. Interestingly, Tauber *et al* (2012) showed that WIN-55 had no effect on MMP-12 expression suggesting that MMP inhibition by WIN-55 may be selective; however this requires further investigation in human OA chondrocytes. Furthermore, the decrease in IL-1β induced pro-MMP-3 and pro-MMP-13 release from chondrocytes demonstrated by WIN-55 in the present study does not necessarily equate to a reduction in active MMPs which degrade proteoglycans and collagens, therefore further studies into MMP activity following WIN-55 treatment is required. It would also be interesting to investigate the effects of WIN-55 on ADAMTS-4 and -5 expression in human OA chondrocytes as these enzymes are important in the breakdown of proteoglycans principally aggrecan, which protects the ECM network from breakdown and thus its breakdown is a pre-requisite for collagen breakdown (Little *et al,* 2007).

Interestingly, studies have demonstrated that MMP-1 and MMP-13 expression requires differential-signalling pathways in articular chondrocytes (Mengshol *et al,* 2000). MMP-13 expression was shown to require NFκB, JNK and p38 phosphorylation and MMP-1 expression was dependent on p38 and ERK1/ERK2 phosphorylation (Mengshol *et al,* 2000). In the present study WIN-55 inhibited both IL-1β induced MMP-3 and -13 mRNA expression and IL-1β induced c-Jun, ERK1/ERK2 and IκB phosphorylation but failed to counteract IL-1β induction of p38 phosphorylation. These finding may indicate that WIN-55 inhibits distinct IL-1β signalling pathways and may therefore display selective inhibition of MMPs, however this requires further investigation as many MMPs in addition to ADAMTSs are involved in the pathogenesis of OA.

In this study WIN-55 was also shown to decrease the mRNA expression of TIMP-1 and -2 in a time and concentration dependent manner. These findings suggest that TIMPs and MMPs share a common expression pathway. Both MMPs and TIMPs share a common AP-1 binding site in their promoters and the AP-1 complex is formed by c-fos and c-Jun heterodimers or c-Jun and c-Jun homodimers (Borden and Heller 1997; Vincenti and Brinckerhoff 2002). Findings presented in this study have shown that WIN-55 significantly reduced IL-1β induced c-Jun phosphorylation therefore possibly reducing the amount of c-Jun available for the formation of AP-1. Although this is a possible mechanism via which WIN-55 mediates its effects, further investigation using transcription factor binding studies is required. Since, PPARs are thought to interact with c-Jun to prevent the formation of the AP-1 complex thus having transcriptionally suppressive activities (Delerive *et al,* 1999) it is necessary to elucidate the effects of WIN-55 on PPARs/c-Jun interactions in human OA chondrocytes. In the present study a pre-treatment of WIN-55 for 48 hours was required to inhibit IL-1β induced IκB, c-Jun and ERK1/ERK2 phosphorylation. These findings suggest that WIN-55 may be acting via an indirect pathway to inhibit IL-1β signalling. In addition, WIN-55 has been shown to increase the mRNA expression of PPARα and δ following 48 hours treatment (Chapter 5). Therefore PPARs may interact with other kinases in addition to c-Jun, thus acting to suppress their phosphorylation and subsequent activation of target genes following IL-1β stimulation, this requires further investigation.

In human OA cartilage, miR-146a may decrease IL-1β induced MMP-13 expression via downregulation of IRAK1 and TRAF6, (Yamasaki *et al,* 2009). Furthermore up-regulation of MMPs in human OA chondrocytes was shown to be associated with demethylation of specific CpG sites in the promoter regions (Roach *et al,* 2005). WIN-55 may also have effects at the DNA level to prevent MMP expression, including induction of histone deacetylases such as SIRTI or modulation micro-RNAs. In order to identify the effects of WIN-55 on micro-RNAs and DNA methylation would require further investigation using *In situ* hybridization and methylation-sensitive restriction enzymes respectively. In addition WIN-55 may affect the binding of AP-1 to consensus sequences in the MMP promoter, effects that have been shown by gel mobility and supershift assays in human synovial fibroblasts and chondrocytes on activation of PPARγ with selective agonists (Fahmi *et al,* 2002; Francois *et al,* 2004). Since WIN-55 is known to bind to PPARγ (O'Sullivan 2007), it is possible that WIN-55 induces similar actions at this receptor thus inhibiting MMP expression.

Cannabinoids have been shown to prevent joint damage *in vivo* and in bovine nasal chondrocytes WIN-55 and HU210 were shown to inhibit IL-1α induced proteoglycan and collagen degradation (Mbvundula *et al,* 2006; Malfait *et al,* 2000; Sumariwalla *et al,* 2004; Zurier *et al,* 1998). These findings suggest that WIN-55 may also prevent cartilage breakdown in human OA chondrocytes via preventing the resorption of collagens and proteoglycans, therefore it would be important to investigate the effects of WIN-55 on ECM molecules, in human OA chondrocytes principally collagen type II and aggrecan.

In the present study activation of individual cannabinoid receptors failed to reduce IL-1β induced MMP-3 and -13 expression in human OA chondrocytes. However when used in combination activation of cannabinoid receptors CB1, CB2 and PPARα, δ and γ reduced IL-1β induced MMP-3 and -13 expression. These findings suggest that WIN-55 may mediated its effects via the activation of multiple cannabinoid receptors, which could be investigated using cannabinoid receptor antagonists. It is possible that WIN-55 may induce its effects in human OA chondrocytes by a yet unidentified cannabinoid receptor as also suggested by others (Selvi *et al,* 2008; Curran *et al,* 2005). In addition different combinations of agonists which target other cannabinoid receptors

were not investigated in this study but have been shown here to be expressed by chondrocytes including GPR55, GPR18 and TRPV1. There may also have effects on MMP-3 and -13 expression. The preliminary data obtained using combinations of cannabinoid receptor agonists on the IL-1β signalling pathways should be interpreted with caution, since these treatments resulted in a significant decrease in cell viability. Furthermore the effects of WIN-55 in addition to selective agonists on the total amount of (unphosphorylated) c-Jun, IκB, p38 and ERK1/ERK2 requires further investigation.

In this study, WIN-55 treatment appeared to affect the cellular localisation and protein expression of CB1, CB2, GPR18, TRPV1 and PPARα and δ in OA chondrocyte cultures. In order to further elucidate the effects of WIN-55 on cannabinoid receptor expression, cellular localisation and activation within OA chondrocytes requires further investigation.

Endogenous cannabinoids AEA and 2-AG bind to cannabinoid receptor CB1 and CB2 and have been identified in the synovial fluid of OA patients (Pertwee 2005; Richardson *et al,* 2008). Modulation of endogenous cannabinoids in the joint may be of therapeutic value in the treatment of arthritis. Since, endogenous cannabinoids including AEA and 2-AG are readily broken down by FAAH and MAGL respectively, inhibition of this process may lead to increased expression of endogenous cannabinoid within the joint (Pertwee 2005), however the effects of endogenous cannabinoids on chondrocyte metabolism requires further investigation.

Although cartilage breakdown is a key pathological feature of OA, other tissues of the joint are also involved including the bone and synovium, which together contribute to the disease progression (Loeser *et al,* 2012). Since cannabinoid receptors are thought to be involved in bone metabolism as shown by *in vivo* animal models (Idris and Ralston 2010) and here it is shown that cannabinoid receptors are expressed by osteocytes in OA bone it is also important to determine the effects of cannabinoids on bone remodelling during OA.

7.2 Conclusions

The present study has shown that cannabinoids may be of therapeutic value in the treatment of arthritis via inhibiting IL-1β induced MMP expression and IL-1β

signalling pathways. Data presented here shows that cannabinoid receptors, are expressed by OA chondrocytes in all zones of articular cartilage and receptor expression of CB1, CB2, GPR55 and PPARα and δ did not appear to be associated with grade of degeneration, suggesting that cannabinoid receptor targeted therapy may be effective even in higher grades of degeneration. However decreases in GPR18 and TPRV1 expression in the deep zone of cartilage and PPARγ expression in osteocytes was associated with grade of degeneration suggesting these receptors may be involved in the pathogenesis of OA.

Furthermore, co-expression of cannabinoid receptors in OA cartilage suggests that WIN-55 may mediate its effects via multiple receptors. The combinational use of cannabinoid receptor agonists for CB1, CB2 and PPARα, δ and γ reduced IL-1β induced expression of MMP-3 and -13 in human OA chondrocytes suggesting that WIN-55 may mediate its effects via activating multiple cannabinoid receptors. Collectively, these findings provide insight into the chondroprotective effects of activation of cannabinoid receptors expressed by chondrocytes and suggest cannabinoids may present a possible therapy in the prevention of cartilage breakdown in OA.

Appendix 1 Human OA Patient Samples

Patient Sample	Gender	Age	Sample ID	Anatomical Compartment	Macroscopic Grade
HC1	Male	64	HC1(1)	Left knee femoral condyle	2
HC2	Female	79	HC2(3)	Posterior condyle	3-4
HC3	Female	73	HC3(3)	Posterior condyle	2
			HC3(4)	Tibia	3
			HC3(6)	Trochlea	3
HC4	Female	67	HC4(1)	Posterior femoral condyle	3
			HC4(2)	Posterior femoral condyle	3
			HC4(3)	Medial femoral condyle	1
			HC4(6)	Medial femoral condyle	3
HC5	Female	57	HC5(1)	Medial femoral condyle	2-3
			HC5(2)	Posterior femoral condyle	0
			HC5(4)	Lateral femoral condyle	2
			HC5(5)	Medial Tibial Plateau	1
			HC5(7)	Femoral trochlea	3-4
HC6	Female	72	HC6(1)	Lateral Tibial Plateau	0
			HC6(2)	Lateral Femoral Condyle	1
			HC6(3)	Medial Femoral Condyle	2

			HC6(4)	Lateral Femoral Condyle	2
			HC6(5)	Femoral Trochlea	3-4
			HC6(6)	Medial Tibial Plateau	3-4
HC7	Male	72	HC7(1)	Posterior femoral condyle	0
			HC7(6)	Medial femoral condyle	4
HC9	Female	82	HC9(1)	Posterior femoral condyle	0
			HC9(3)	Posterior femoral	2
			HC9(7)	condyle Medial femoral condyle	4
HC10	Male	73	HC10(1)	Trochlear	0
			HC10(6)	Medial femoral condyle	4
HC11	Male	72	HC11(1)	Posterior condyle	0
			HC11(2)	Tibia	2-3
			HC11(3)	Posterior condyle	3
			HC11(4)	Lateral condyle	3
			HC11(6)	Medial condyle	4
HC12	Female	67	HC12(5)	Lateral tibial plateau	4
HC13	Female	83	HC13(2)	Medial tibial condyle	2
			HC13(4)	Lateral posterior condyle	3
HC14	Female	89	HC14(3)	Trochlea	2
HC15	Female	74	HC15(3)	Trochlea	3
			HC15(4)	Medial femoral condyle	3
HC16	Male	81	HC16(2)	Posterior femoral condyle	1
			HC16(4)	Posterior femoral condyle	3
			HC16(6)	Tibia	3
HC17	Female	60	HC17(1)	Medial femoral condyle	0
			HC17(4)	Trochlea	2
			HC17(6)	Posterior condyle	3
HC18	Male	65	HC18(1)	Laterial posterior femoral condyle	1

			HC18(4)	Lateral tibial condyle	2-3
HC19	Female	57	HC19(1)	Lateral femoral condyle	0
			HC19(2)	Posterior condyle	0
			HC19(3)	Tibia	2
HC20	Male	77	HC20(1)	Posterior condyle	0
HC21	Female	64	HC21(4)	Lateral femoral condyle	2
			HC21(5)	Medial femoral condyle	2-3
HC22	Male	59	HC22(4)	Posterior femoral condyle	3
HC23	Female	73	HC23(4)	Posterior condyle femoral medial	2

HC: Human cartilage

Appendix 2 Primer Efficiency's

Taqman Gene Expression Assay	Assay ID	Threshold	Amplification Factor	Primer efficiency (%)
GAPDH	Hs9999905_m1	0.5	1.95	95
18S	Hs99999901_s1	0.75	1.93	93.50
MMP-3	Hs00968305_m1	0.5	1.97	96.90
MMP-13	Hs00233992_m1	0.5	1.99	98.68
TIMP-1	Hs00171558_m1	0.75	2	99.7
TIMP-2	Hs00234278_m1	0.75	1.95	95.30
IL-8	Hs00174103_m1	0.5	1.94	94.11
NGF	Hs01113193_m1	0.5	1.80	80.37
Substance P	Hs00243225_m1	0.5	Not Determined	
PPARα	Hs00947539_m1	0.5	1.74	74.03
PPARδ	Hs00606407_m1	0.5	1.97	97.49
PPARγ	Hs01115513_m1	0.5	Not Determined	

Appendix 3 Automatic Wax Processing Schedule

50% IMS	90 min
70% IMS	60 min
99% IMS	60 min
99% IMS	60 min
99% IMS	60 min
99% IMS	90 min
99% IMS	90 min
SUBX	90 min
SUBX	90 min
SUBX	90 min
Molten Wax	90 min
Molten Wax	120 min

Appendix 4 Suppliers Details

Abcam	Cambridge, UK
Acris Antibodies	Herford, Germany
Ambion	Paisley, UK
Bioline	London, UK
Cayman Chemical	Tallin, Estonia
Fisher Scientific	Loughborough, UK
Graphpad Software Inc	San Diego, USA
Invitrogen	Paisley, UK
JeioTech	Jencons, East Grinstead, UK
Lecia	Milton Keynes, UK
Life Technologies	Paisley, UK
Nunc	(Fisher Scientific)
Olympus Corporation	Tokyo, Japan
Peprotech	London, UK
PerkinElmer	Massachusetts, USA
Promega	Southampton, UK
Qiagen	Crawley, UK
R&D systems	Minneapolis, USA
Sigma-Aldrich	Dorset, UK
StatsDirect Ltd	Altrinham, UK
Thermo Scientific	Hemel Hampstead, UK
Tocris	Bristol, UK
Vector Laboratories	Peterborough, UK

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