Investigation into the immuno-therapeutic potential of melanocortin peptides on activated chondrocytes

Magdalena Krasimirova Kaneva

School of Life Sciences

Magdalena Krasimirova Kaneva

Investigation into the immuno-therapeutic potential of melanocortin peptides on activated chondrocytes

This thesis is presented for the degree of Doctor of Philosophy of University of Westminster

2011

Department of Human and Health Sciences

School of Life Sciences

University of Westminster

London, United Kingdom

DECLARATION

I declare that this thesis is the result of my own research, and as its main content it contains work that has not been previously submitted for degree at any tertiary educational institution.

Magdalena K. Kaneva

I dedicate this Ph.D. dissertation
to my teachers and parents

Посвещавам тази докторска дисертация
на моите учители и родители

TABLE OF CONTENTS

ABSTRACT

Melanocortin peptides are endogenously produced peptides originating from the post-translational processing of the pro-opiomelanocortin hormone (POMC), exerting their effect by binding to class A G-protein-coupled 7 transmembrane domain receptors, positively coupled to adenylate cyclase. To date five melanocortin receptors have been identified and termed MC_1 to MC_5. MC_1 and MC_3 have previously been proposed to exert anti-inflammatory effects by modulating the host inflammatory response. The expression and the functional activity of both receptors was identified and confirmed in the C-20/A4 chondrocyte cell-line, isolated primary bovine and *in situ* bovine articular chondrocytes.

Pro-inflammatory cytokines including IL-1β, IL-6, IL-8, TNF-α, produced by activated articular chondrocytes significantly up-regulate matrix metalloproteinases (MMPs) gene expression, and inhibit the chondrocyte's compensatory synthesis pathways required to restore the integrity of the degraded extracellular matrix (ECM). Human C-20/A4 and primary bovine articular chondrocytes were found to produce CC and CXC chemokines, which induced the release of matrix degrading enzymes and activated cell apoptotic pathways. TNF-α significantly up-regulated the expression of pro-inflammatory cytokines and chemokines IL-1β, IL-6, IL-8, MCP-1 and MMP1 and 13 from C-20/A4 cell line and freshly isolated primary bovine articular chondrocytes. An effect attenuated in the presence of α-MSH and D[TRP]^8-γ-MSH. The $MC_{3/4}$ antagonist SHU9119 blocked the effects of D[TRP]^8-γ-MSH but not α-MSH.

TNF-α (60.0 pg/ml) stimulation caused ~30% cell death and was partially, but significantly inhibited by treatment of the cells with the melanocortin peptides. The anti-inflammatory and chondroprotective effect of melanocortin peptides were then tested on *in situ* bovine articular chondrocytes, injured by a single blunt impact delivered by a drop tower. The mechanical injury caused significant cell death and up-regulation of the pro-inflammatory cytokines IL-6 and IL-8, which were significantly reduced on pre-treatment of cartilage explants with melanocortin peptides.

Modulation of pro-inflammatory pathways and inflammation-modulated cartilage destruction with subsequent chondrocyte apoptosis appears to be logical development in the potential medical therapy of OA. The small molecular weight of melanocortin peptides should facilitate the absorption from the GI tract and the movement to the cartilage matrix, which together with creative drug delivery methods might potentially prove to be potent therapeutic agents in the future.

ACKNOWLEDGEMENTS

ABBREVIATIONS

Abbreviation	Full name
AA	Acrylamide
ACTH	Adrenocorticotrophic Hormone
Anti-CCP	Anti-Cyclic Citrulinated Peptide
AP-1	Activating Protein-1
APS	Ammonium Persulphate
ATP	Adenosine 5'-Triphosphate
A_{xxxnm}	Absorbance at xxx nm
BA	Bis–Acrylamide
bp	Base pair
BSA	Bovine Serum Albumin
CASP1	Caspase-1
cDNA	Complementary DNA
CLC	Cardiotropin-like cytokine
CNTF	Cilliary neurotropic factor
COL1A2	Collagen type I
COL2A1	Collagen type II
CO_2	Carbon dioxide
COX–1/2	Cyclooxygenase 1 and 2
$cPLA_2$	Cytoplasmic Phospholipase A_2
CREB	cAMP Response Element-Binding protein
CS	Chondroitin sulphate
CT	Cardiotropin
D	Aspartate residue
DD	Death Domain

DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleoside 5'–triphosphate
DTT	Dithiotreitol
ECSIT	Evolutionarily conserved signalling intermediate in toll pathways
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immuno assay
ELISA	Enzyme–linked immunosorbant assay
ERK	Extracellular signal-regulated kinase
EtOH	Ethanol
ETS	E twenty six
FasL	Fas Ligand
FCS	Fetal calf serum
g	Gram
g	Gravity acceleration
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde–3–phosphate dehydrogenase
GI	Gastrointestinal
GPCR	G–protein coupled receptor
GRE	Glucocorticoid Response Elements
h	Hour
HPA axis	Hypothalamo–Pituitary–Adrenal axis
HRP	Horse-Radish Peroxidase
Ig	Immunoglobulin
IgG	Immunoglobulin g
IKK	IkappaB Kinase

IL–10	Interleukin 10
IL–1R	Interleukin 1 receptor
IL–1RA	Interleukin 1 receptor antagonist
IL–1β	Interleukin 1 beta
IL–6	Interleukin 6
IL–8	Interleukin 8
INF–γ	Interferon gamma
IRAK	IL-1 Receptor-Associated Kinase
IκB	Inhibitor of kappa B (Inhibitor of NFκB)
JNK	c-Jun N-terminal Kinase
kb	Kilo bases
kDa	Kilo Dalton
kg	Kilogram
KS	Keratan sulphate
L–Arg	L–Arginine
LIF	Leukaemia Inhibitory Factor
LPS	Lipopolysaccharide
LTB$_4$	Leukotriene B$_4$
LTC$_4$	Leukotriene C$_4$
LTD$_4$	Leukotriene D$_4$
M$\varnothing$	Macrophage
mAb	Monoclonal antibody
MAPK	Mitogen-Activated Protein Kinase
MAPKK	MAPK Kinase
MAPKKK	MAPKK Kinase
MC	Melanocortin receptor

MCP–1	Monocyte chemoatractant protein 1
mg	Milligram
min	Minute
ml	Millilitre
mRNA	Messenger ribonucleic acid
MSU	Monosodium urate
MT-MMP	Membrane Type-Matrix Metalloproteinase
NF-κB	Nuclear Factor-kappa B
ng	Nano gram
NIK	NFκB Inducing Kinase
nm	Nanometer
NSAID(s)	Non–Steroidal Anti–Inflammatory drug(s)
OA	Osteoarthritis
OD	Optical Density
p	Probability value
pAb	Polyclonal Antibody
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PGD$_2$	Prostaglandin D$_2$
PGE$_2$	Prostaglandin E$_2$
PGG$_2$	Prostaglandin G$_2$
PGH$_2$	Prostaglandin H$_2$
PGES	Prostaglandin E Synthase
pI	Isoelectric Point
PLA$_2$	Phospholipase A$_2$
POMC	Pro–opiomelanocortin

RA	Rheumatoid Arthritis
RF	Rheumatoid Factor
RIP	Receptor Interacting serine-threonine Protein kinase
RNA	Ribonucleic Acid
RNase	Ribonuclease
ROS	Reactive Oxygen Species
rpm	Revolutions per minute
SBE	STAT-Binding element
SD	Standard deviation
SDS-PAGE	Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis
sec	Seconds
SEM	Standard Error of Means
STAT	Signal Transducer and Activator of Transcription
TAE	Tris-Acetate-EDTA
TBE	Tris Borate Ethylene–(2,2')–diamine–tetracetic acid
TCA	Trichloroacetic Acid
TEMED	N,N,N',N'-Tetramethyl-1,2-diaminoethane
TLR	Toll-like Receptor
TMD	Transmembrane Domain
TNF–α	Tumor Necrosis Factor alpha
TNFR	TNF-α Receptor
TRADD	TNF-α Receptor Associated Death Domain
TRAF	TNF-α Receptor Associated Factor
Tris	Tris(hydroxymethyl)aminomethane
TxA$_2$	Thromboxane A$_2$

TYK2	Tyrosine Kinase-2
U	Units
UV	Ultraviolet
v/v	volume per volume
w/v	weight per volume
α-MSH	Alpha–melanocyte stimulating hormone
β-MSH	Beta–melanocyte stimulating hormone
γ_2–MSH	Gamma–2 melanocyte stimulating hormone
μg	Microgram
μl	Microliter

INDEX TO FIGURES

INDEX TO TABLES

PUBLICATIONS

Papers

GETTING, S. J., RIFFO-VASQUEZ, Y., PITCHFORD, S., **KANEVA, M.**, GRIECO, P., PAGE, C. P., PERRETTI, M. & SPINA, D. 2008. A role for MC3R in modulating lung inflammation. *Pulm Pharmacol Ther,* 21**,** 866-73.
(http://www.ncbi.nlm.nih.gov/pubmed/18992358)

Abstracts

M. KANEVA, I. LOCKE, M. KERRIGAN, S. GETTING. 2011. α-MSH and DTRP^8-γ-MSH inhibit pro-inflammatory cytokine release from stimulated primary bovine chondrocytes. 10th World Congress on Inflammation, Paris, France
(https://b-com.mci-group.com/Abstract/Statistics/AbstractStatisticsViewPage.aspx?AbstractID=62455)

M. K. KANEVA, M. J. Kerrigan, I. C. Locke and S. J. Getting. 2011. MC$_1$ and MC$_3$ activation inhibits caspase-3 production and activity, promotes cell-viability, IL-10 and HO-1 release from human C20/A4 chondrocytes. University of Oxford, *Proc Physiol Soc*, PC77
(http://edition.pagesuite-professional.co.uk/launch.aspx?referral=other&pnum=&refresh=2Hz1S09jp6J0&EID=5f950d03-be13-42cb-9c60-6d5db925019d&skip=) p. 200

KANEVA, M., KERRIGAN, M., LOCKE, I. C. & GETTING, S. 2010. α-MSH and dTRP^8-γ-MSH inhibit TNF-α induced MMP1 and 13 expression in human C-20/A4 chondrocytes. *Proc British Pharmacological Society,*
(http://www.pa2online.org/abstracts/vol8issue1abst125p.pdf).

QUSOUS, A., **KANEVA, M.**, GETTING S. J., AND KERRIGAN M. J. P. 2010. A13/BACII, a novel bovine chondrocytic cell line with differentiation potential. *In: Combined Meeting of Orthopaedic Research Societies (CORS)*, Kyoto, Japan.

KANEVA, M., KERRIGAN, M., LOCKE, I. C., GETTING, S. J. 2010. MC3R agonist dTRP^8-γ-MSH inhibits pro-inflammatory cytokines from TNF-α stimulated C-20/A4 chondrocytes, an effect blocked by the MC3R antagonist SHU9119. University of Manchester, *Proc Physiol Soc 19*, PC256.
(http://www.physoc.org/custom2/publications/proceedings/archive/article.asp?ID=Proc%20Physiol%20Soc%2019PC256).

KANEVA, M., KERRIGAN, M., LOCKE, I. C. & GETTING, S. 2009. Melanocortin peptides modulate pro-inflammatory mediator release from TNF-α stimulated C-20/A4 cells. *Proc British Pharmacological Society*
(http://www.pA2online.org/abstracts/Vol7Issue4abst126P.pdf).

KANEVA, M., KERRIGAN, M., LOCKE, I. C., GETTING, S. J. 2009. Pro-inflammatory mediator release from TNF-α stimulated C-20/A4 cells results in Col II degradation. BORS, Newcastle, PO136

KANEVA, M., KERRIGAN, M., LOCKE, I. C. & GETTING, S. 2008. A role for melanocortin peptides in modulating oxidative stress induced inflammation in A549 cells. *Proc British Pharmacological Society*
(http://www.pA2online.org/abstracts/Vol6Issue4abst020P.pdf).

Chapter 1
Introduction

1.1   Cartilage.

Mammalian cartilage is a complex and developmentally critical tissue that has been historically defined as a vertebrate tissue component of the skeletal system, fundamentally different in structure and function from bone tissue. It functions as an internal cellular support tissue and is composed of fibrous proteins and mucopolysaccharides (Cole and Hall, 2004). Three different types of cartilage exist in mammals; each differing in the arrangement and proportion of extracellular fibrous (collagen) or non-fibrous (proteoglycans) macromolecules within the extracellular matrix (ECM), in the distribution and morphology of the only resident cell type – the chondrocyte, and their anatomical location and functions within the body (Hall, 1998).

Hyaline cartilage, also wildly known as articular cartilage, is the most studied cartilaginous tissue. It is particularly important due to its function in development, as it lays down the scaffold for the future development of most of the key bones within the body, and due to its preservation at the joint interfaces, where it creates a smooth surface for frictionless joint movement. It is also the primary tissue affected by arthritic pathologies (Hollander *et al*., 2010).

In hyaline cartilage, the proportion of collagen to proteoglycans, is such that under polarized light microscope the tissue appears glassy, with translucent blue-white appearance in early age, turning pale and opaque with aging (Van der Korst *et al*., 1968, Hall, 1998). Articular cartilage constitutes the temporary embryonic skeleton, before its replacement with bone. It can also be found in the epiphyseal plates between the diaphysis and epiphysis of growing long bones, where it is responsible for the longitudinal growth of the bone (Martel-Pelletier and Pelletier, 2010). In adults, this type of cartilage is present in the articulating surfaces of articular joints, in the wall of the large respiratory passages, such as the nose, larynx, trachea, bronchus, and at the ventral ends of the ribs that move with the sternum. The tissue is avascular, alymphatic and aneural and populated only by chondrocytes, which are responsible for the synthesis, exportation and degradation of ECM components – proteoglycan, fibrous collagen (type I, type II, and small amounts of types VI, IX and XI) and other proteins. The surface areas of the articular cartilage can withstand tensile forces arising from joint movement

but its principle adaptation is to withstand compressive forces (Martel-Pelletier and Pelletier, 2010).

Fibrocartilage, or white fibrocartilage, is another type of cartilage found mainly in the invertebrate disks of the spine, the attachment of some ligaments to the bones, in the pubic symphysis, but also in knee, jaw and healing bones (Benjamin and Evans, 1990, Benjamin and Ralphs, 1998). Its main function is to withstand compression forces. Fibrocartilage is a very dense connective tissue, composed mainly from collagen type I (Eyre and Muir, 1975), relatively little proteoglycan (Gillard *et al.,* 1979, Koob and Vogel, 1987) and some elastic material (Buckwalter *et al.,* 1976). It develops by metaplasia from pre-cartilage, hyaline cartilage and particularly from fibrous tissue, and these features precede the appearance of cartilage cells (Benjamin and Evans, 1990).

The third type of cartilage is the elastic cartilage, or yellow fibrocartilage. Elastic cartilage is composed of elastic fibres as well as collagens and proteoglycans and is mainly found in the auricle of the ears, in the Eustachian tubes and epiglottis. It is described as rubber-like, highly and reversibly deformable. Elastic cartilage chondrocytes highly resemble those in hyaline cartilage, but these synthesize elastin in addition to the other matrix components (Hall, 1998).

1.1.1   Articular Cartilage – structure and function.

The mechanical resistance and flexibility of cartilage occurs because of the four components of the extracellular matrix and their properties – proteoglycans, collagen, interstitial fluid and chondrocytes. Proteoglycans are proteins composed of highly negatively charged glycosaminoglycans (GAGs), covalently attached through O-glycosidic bond at their reducing ends to the core protein, commonly chondroitin sulphate (CS) or keratan sulphate (KS) (Jackson *et al.,* 1991). Aggrecan, one of the largest proteoglycans and the major one in articular cartilage, is a large chondroitin sulphate proteoglycan composed of a large number of GAG monomers and represents the bulk of the proteoglycans in cartilage (Watanabe *et al.*, 1998). It interacts with the hyaluronan and other link proteins to form macromolecular complexes, trapped by the collagen network and gives cartilage its capacity to resist compression through hydration (Jackson

et al., 1991). Decorin, biglycan and fibromodulin, are smaller proteoglycans, rich in leucine, representing around 20-25% of all proteoglycans in cartilage, and are associated with the proteoglycans and the 'ground substance' – the main components of which are large carbohydrates and other proteins (Hardingham and Fosang, 1992). A central component of the proteoglycan aggregates is hyaluronan, which is a non-sulphated glycosaminoglycan and is present in relatively small amounts in cartilage. It is, however, an important component of the matrix, as CD44, expressed on the chondrocytes cell surface, bind to hyaluronan, thus embedding the chondrocyte within the extracellular matrix (Figure 1.1).

Collagen fibres are the principle component of connective tissues and are composed of bundles of fibrils, which are made of stacked molecules of helical polypeptide chains. Fourteen different types of collagens exist, which vary both in size and in configuration (Eyre, 2004). Articular cartilage is mainly composed of a unique type of collagen, type II, which is structurally characterised by the aggregation of large fibrillar homotrimer complexes, with three identical α-chains peptides (formed by the repeating tri-peptide sequence Gly-x-y) each adopting left-handed helical conformation (Hall, 1998) with the three chains twisted together in a right-handed helix. Collagen type II in cartilage forms a 3D fibrillar network of rope-like molecular aggregates, which together with the proteoglycans is essential for maintaining the tissue volume, shape and tensile strength (Eyre *et al.*, 1992) . Collagen II represents >95% of all the collagens present in the cartilage. Other collagens found in cartilage include type I, type IX, type X, which play an essential role in the matrix organization. Collagen type IX is a non-fibril, highly glycosilated collagen that covalently links to type II collagen fibrils, thereby helping in the interweaving and cross-linking of the collagen fibrils and trapping the hydrated proteoglycans. Collagen type IX is additionally regarded as a proteoglycan due to a CS chain attached and is thought to provide lateral strength and flexibility (Hall, 1998).
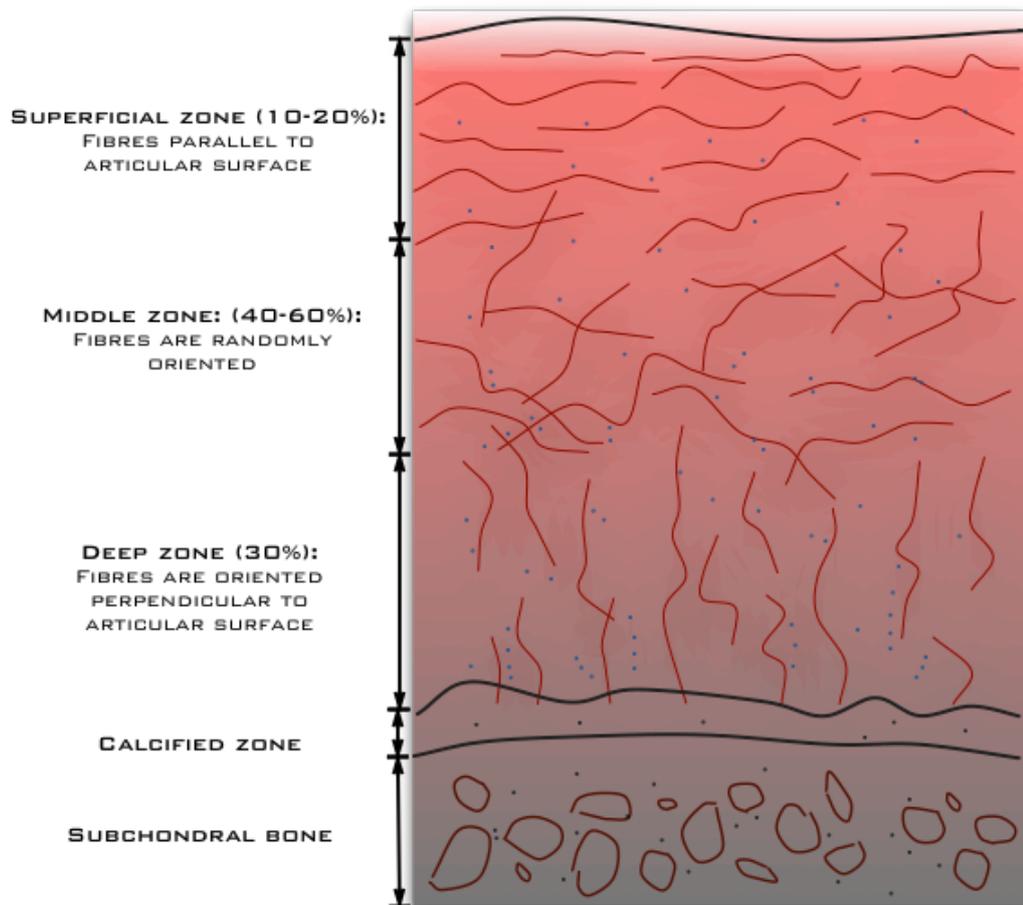
Figure 1.1 Articular cartilage zones and zonal orientation of collagen type II fibrils. A simplified diagram of a perpendicular section of articular cartilage and underlying bone, showing the heterogeneity of the cartilage matrix. Arcades of collagen bundles originating in the calcified zone and binding the non-calcified cartilage are extending towards the articular surface and then curving back down. In sequence from the articular surface to the subchondral bone, the collagen bundles are tangential, radial and perpendicular. The collagen fibres are continuous with those in the calcified layer of cartilage but not with underlying subchondral bone. The morphology of the collagen-fibril network influences the local stresses and strains in the articular cartilage.

The organisation and distribution of ECM macromolecules and chondrocytes differs significantly within the tissue and can be separated into four distinct regions. The first region located at the articular surface of the cartilage is the *superficial* zone. It is composed of thin, tangential, tight collagen fibrils, mainly

parallel to tangential stress and is associated with low concentration of aggrecan and high concentrations of decorin. The *middle zone* contains thick radially, randomly oriented, looser bundles of collagen, whilst the *deep zone* (also called radial zone) contains perpendicularly oriented collagen fibrils and the *calcified cartilage zone (*located between the deep zone and the subchondral bone) serves as a mechanical buffer (Muir *et al*., 1970, Wilson *et al*., 2004). It has been observed that the cell density progressively decreases from the superficial zone to the deep zone, whereas relative to collagen, cell volume and the proteoglycan content increase.

Within cartilage, there are not only topographical variations in the quantity of proteoglycan, but also the type, thus contributing to the physical properties of the matrix. The GAG side chains of proteoglycans are sulphated and responsible for a fixed negative charge density, which binds mainly sodium, but also other mobile cations (Lesperance *et al*., 1992*)*. This process, together with the quality and quantity of the collagen network is deterministic for the osmotic pressure of the extracellular fluid around chondrocytes. Extracellular osmolarity in healthy articular cartilage ranges between 350 and 480 mOsm.kg^{-1} H_2O, hereafter termed 'mOsm' (Maroudas, 1976) and is highly dynamic, in response to changes in matrix hydration (Urban, 1994). During osteoarthritis, the collagen matrix degrades and the concentration of GAG diminishes in a disease progression-dependent way, leading to decreased osmolarity to between 280-350 mOsm (Maroudas, 1976, Bush and Hall, 2001a, Bush and Hall, 2005).

The proteoglycan molecules (3–10 % of tissue on wet weight basis) are highly negatively charged with sulphate, SO_4^{2-} and carboxyl (COO^-) functional groups, which, together with the positively charged cations drawn from the synovial fluid, cause the osmotic pressure in the matrix to increase and consequently leads to water inhibition and tissue inflation. However, within the cartilage, the expansion of the proteoglycans is limited by the tension of the collagen matrix (15 – 30 % of tissue on wet weight basis), which restricts proteoglycans hydration to only 20 % of their actual capacity, and thus creates swelling pressure within the cartilage, contributing to its compressibility (Hall, 1998). When cartilage is loaded, during normal daily activities, water is extruded and the PGs are further compressed, but upon removal of the load (resting) water is imbibed in the tissue, together with

essential nutrients, until the swelling pressure of the aggrecan is balanced again by the tensile force of the collagen matrix. The interstitial fluid (65 – 80 % of the tissue) is what forms the aqueous environment in which the chondrocytes reside. It supplies the cells with substrates, nutrients and matrix biosynthesis precursors, and also allows for metabolic by-products to be eliminated from the tissue.

1.1.2 Chondrocytes.

Connective tissues are highly active living tissues with capacity to support and resist mechanical forces, crucial for the musculoskeletal system. The cells of connective tissues continually produce and maintain appropriate macromolecules that give the tissue its required mechanical properties. This occurs by synchronised equilibrium of anabolism and catabolism of those macromolecules (Gardner, 1992).

Chondrocytes are present at a very low density of ~ 1-5 % of the adult cartilage tissue, with approximately 1.0 x 10^3 cells per mm^3 (human femoral head), and depending on the location, little or no cell division (Hall, 1998). Although the role of the chondrocytes has historically been marginalized because they do not play a direct mechanical role, they are the only existing units capable of producing, repairing or degrading the matrix. Additionally, chondrocytes 'sense' physical changes in their environment and this determines matrix metabolism and ultimately the mechanical characteristics of the tissue, the osmotic composition of the interstitial fluid, cell-matrix interactions and the rate of diffusion of substances between the synovial fluid and the interstitial fluid.

Chondrocytes in cartilage are heterogeneous, and this is related to the topography within different zones of the tissue (Stockwell and Meachim, 1973, Hall, 1998). In addition chondrocytes can exist as single units or in groups of up to six cells. Differences in the morphology of zonal chondrocytes can be easily observed. Chondrocytes from the superficial zone occur singly or in pairs, are small (8–15 μm in diameter), sparsely distributed and ellipsoid in morphology. In the middle zone, chondrocytes are oval-shaped approximately 15–25 μm in diameter and are arranged radially, singularly or in pairs (Stockwell, 1971, Hall *et al*., 1996, Hall, 1998). Chondrocytes become more oval in the deep zone, where

they are found in small groups or short columns of about 3–5 cells (Hall, 1998, Hall *et al*., 1996).

Early studies have identified that the matrix immediately surrounding the chondrocyte in hyaline cartilage is different and more specialised than the intercellular/interterritorial matrix, which led to the identification of a primary functional and metabolic unit termed 'chondron' (Poole *et al*., 1992). The chondron consists of one or several chondrocytes, the pericellular, or "lacunar" matrix, which is rich in proteoglycans and possibly hyaluronic acid, relatively no collagens, and the pericellular rib, which includes types VI, II and IX collagen (Figure 1.2). It has been hypothesized that the microenvironment, surrounding the chondrocytes within their chondrons, serves to protect the chondrocytes by dampening the physicochemical, osmotic and mechanical changes that happen during dynamic loading (Poole *et al*., 1992). Continuous with the pericellular matrix is the territorial matrix, which is composed of basket-like network of cross-linked collagen fibrils, forming a capsule around the chondron, which the chondrocyte contacts through numerous cytoplasmic processes, rich in microfilaments. The pericellular collagens diverge and converge at the superficial pole of the chondron capsule, forming a pericellular channel between the interior of the chondron and the intercellular matrix. It has been suggested that, during compressive pressure to tissue, these "pericellular channels" underline a mechanism for the chondrocytes to sense changes in the osmotic pressure and therefore regulate matrix metabolism (Hall *et al*., 1996).
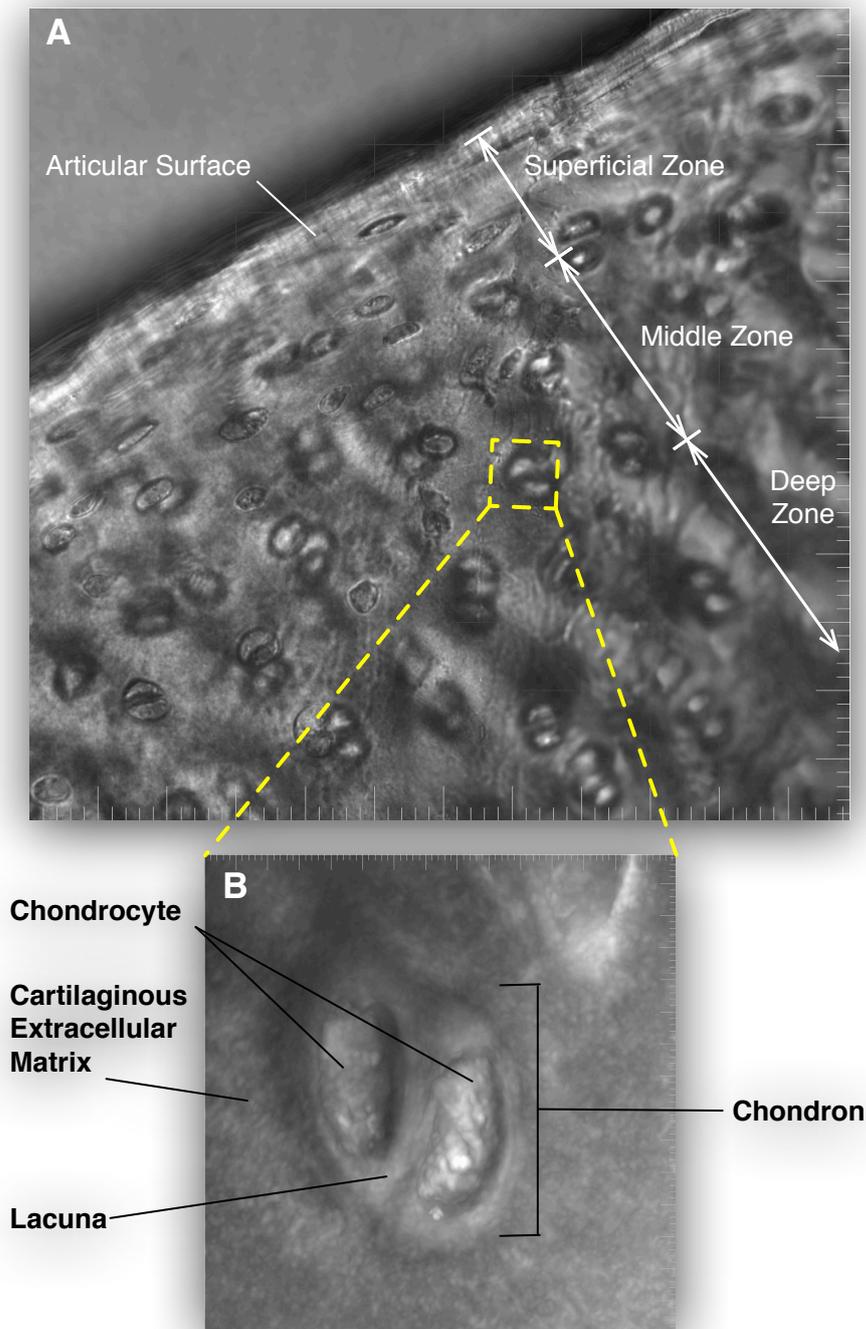
Figure 1.2 High power phase contrast confocal micrograph of articular hyaline cartilage excised from adult (18-24 months old) bovine 'knee' joint.

Articular cartilage has a complex internal structure. Composed of four poorly demarcated zones, the most superficial, uppermost zone forms the gliding surface and is in contact with the synovial cavity of the joint. Small elliptical chondrocytes are oriented parallel to the surface; chondrocytes in deeper zones are larger, more rounded, and arranged in vertical columns. The term chondron encompasses the chondrocyte and its pericellular and territorial matrix. Image was taken using Leica TCS SP2, x630 magnification.

1.2   Arthritides.

1.2.1   Rheumatoid Arthritis (RA).

Rheumatoid arthritis is a complex systemic, inflammatory pathology, primarily affecting the joints, with cardiovascular problems being causal to 40% of the morbidity associated with this pathology. RA is one of the most common of the arthritides, affecting middle-aged males and females, with a marked female bias (approx. 2.5 times; (Lee and Weinblatt, 2001) as well as children (in the case of juvenile variant). Rheumatoid arthritis has acute or insidious onset, and typically, but not exclusively, affects the joints in a symmetrical manner the radiocarpal and metacarpophalangeal, proximal interphalangeal joints of the hands and feet and occasionally large joints (Lee and Weinblatt, 2001). Cardinal features of RA are early morning stiffness and pain, which can last for more than 1 h, joint swelling and deformity, synovial thickening, reduced range of motion of the affected joint and systemic features including flu-like symptoms, fatigue, fever, depression and weight loss (Lee and Weinblatt, 2001).

The exact pathology of RA is still not understood, but it is generally accepted to be a disease of autoimmune origin, resulting in a continual immunological reaction, mostly directed against joint tissues, but may also have manifestations as a more systemic syndrome involving a variety of organs (formation of rheumatoid nodules in skin, salivary inflammation, pulmonary fibrosis, pericardial inflammation, myocarditis, mononeuritis, anemia, thrombocytosis, vasculitis (Lee and Weinblatt, 2001). Characteristic findings in RA joints include synovial inflammation with an accumulation of inflammatory cells, increased synovial levels of pro-inflammatory cytokines, and the formation of a characteristic area of granulation tissue known as a pannus, which is formed from both synovial cells and the cellular infiltrate, and appears to contribute to the destruction of the underlying cartilage (Lee and Weinblatt, 2001).

Quantitative analyses have shown that a wide variety of pro-inflammatory cytokines are present in inflamed synovial tissue and are produced in moderate to high concentrations in RA. Tumor necrosis factor (TNF)-α and Interleukin (IL)-1β are both present in high concentrations in affected synovial fluid and synovial

tissue (Chu *et al.*, 1991, Wood *et al.*, 1992, Lee and Weinblatt, 2001), where they act as potent stimulators of proliferation, matrix metalloproteinase expression, up-regulation and release of pro-inflammatory cytokines and prostaglandin production (Lee and Weinblatt, 2001). Previous studies have identified IL-1β as the main mediator for the initiation of the proteolysis in rheumatoid arthritis and in other inflammatory joint diseases (Dayer *et al.*, 1979, Mizel *et al.*, 1981, Krane, 1982, Baracos *et al.*, 1983, Wood *et al.*, 1992). The enhanced secretion of collagenases and prostaglandin E_2 (PGE_2), by synovial cells, is considered a factor for the degenerative progression that is clinically observed. TNF-α, a potent stimulator of collagenase and PGE_2 production in synovial cells (Choi *et al.*, 2009, Chu *et al.*, 1991, Kunisch *et al.*, 2009), is also implicated in the disease progression with production in far larger quantities than IL-1β under some circumstances *in vivo*.

Whilst RA is generally classified as an autoimmune disease, the mechanisms involved and the significance of auto-antibodies routinely detected in RA patients, remain uncertain. The main auto-antibodies, detected in roughly 70–80 % of RA patients (Avouac *et al.*, 2006), are rheumatoid factor (RF) and anti-cyclic citrulinated peptide (anti-CCP), antibodies directed against the fragment crystallizable (Fc) region of IgG antibodies and proteins containing citrulline (a modified form of the amino acid arginine), respectively (van Venrooij *et al.*, 2008, van Venrooij and Zendman, 2008, Getting *et al.*, 2009). It is suggested that these antibodies may be involved in RA via the formation of immune complexes, which together with their target antigens result in complement activation, the release of other pro-inflammatory mediators from the synovium, and leukocyte recruitment and infiltration into the joint space (Lee and Weinblatt, 2001, Brennan and McInnes, 2008, van Venrooij and Pruijn, 2008).

1.2.2   Metabolic Arthritis/Gouty Arthritis.

Gouty arthritis is a chronic joint disease caused by the deposition of monosodium urate monohydrate (MSU) crystals in the joints (McCarty *et al.*, 1966, McGill and Dieppe, 1991, Chilappa *et al.*, 2010). It is an inflammatory arthritis that predominantly affects middle-aged/older individuals, with incidence significantly higher in men than in pre-menopausal women (Getting and Perretti, 2001). Gouty

arthritis can occur in any synovial joint, but mainly manifests as a monoarthritic attack in the smaller joints, particularly the metatarsophalangeal joint, with clinical manifestation of symptoms including erythema, edema of the affected joints and severe pain (McGill and Dieppe, 1991). Gouty arthritis occurs predominantly as a consequence of hyperuricaemia (increased levels of plasma urate; a product of purine metabolism), although other factors including joint trauma and local temperature may be additionally involved (Heuckenkamp *et al.*, 1982, Fokter and Repse-Fokter, 2010). When plasma solubility of urate is exceeded, MSU crystals deposit in a range of soft tissues and joints, resulting in gouty arthritis.

The mechanisms of urate-induced inflammation are not completely understood. Gouty arthritis seems to be initiated primarily by an innate immune response, particularly due to the intense infiltration of blood-borne neutrophils and monocytes into the joint space (Terkeltaub *et al.*, 1991a,b; Villiger *et al.*, 1992), resulting from the elevated secretion of pro-inflammatory mediators, such as IL-1β, IL-6, IL-8, TNF-α and thus the increased expression of endothelial cell adhesion molecules (di Giovine *et al.*, 1991, Villiger *et al.*, 1992). Recent work has shown that toll-like receptors TLR2 and TLR4, present on phagocytes and other cells contained in the joint environment, recognize the MSU crystals, which upon binding to the receptors, induce generation of these pro-inflammatory intermediaries, thereby providing a possible mechanism for disease instigation and progression (Cronstein and Terkeltaub, 2006).

1.2.3 Osteoarthritis (OA).

Osteoarthritis is a slowly progressive, degenerative, multifactorial disease characterized by gradual degradation and loss of articular cartilage, and resulting in loss of joint mobility and pain (Goldring, 2000a, Aigner and Stove, 2003, Goldring and Goldring, 2007). OA has been classified as a late-onset, complex disease, which affects over 100 million individuals all over the world (Iliopoulos *et al.*, 2008). It is the most common of the arthritides, and is largely a disease of the elderly and middle aged. In theory, OA can affect any synovial joint in the body, but OA lesions are typically localized to the weight-bearing regions of the larger joints (the hip, knee and spine), and later in those of the hand (Buckwalter and Mankin, 1997).

The exact pathogenesis of osteoarthritis is not fully understood with obesity, age, abnormal joint loading and sport injuries all being risk factors (De Bri *et al.*, 1998), but OA is more than just the result of "wear and tear" (Aigner *et al*., 2004b). Abnormal joint loading causes changes in physiology of the chondrocyte, which mediate the biosynthesis of ECM macromoledules. It is now generally accepted that the articular chondrocytes play an important role in the initiation and progression of OA (Goldring, 2000b).

It is not yet known what is the exact role of the chondrocyte in the initiation and progression of osteoarthritis, due to the complexity of the disease process and its high localization of focal lesions. Dieppe, 1994 hypothesized that the initiation and the progression of osteoarthritis within individual joints, and even the disease distribution between the joints, may be ascertained by the specific balance between local and systemic factors (Dieppe and Kirwan, 1994). In the cases where, for example, excessive mechanical loads cause micro-damage to cartilage, chondrocytes might not be involved directly. The injury might initiate a sequence of cartilage ECM damage, before the obvious changes to chondrocyte physiology are detected. It has been observed that there are focal defects (peak loading location) in osteoarthritis of the hip and knee, suggesting that in some cases mechanical factors are not only an important risk factor for the development of changes to chondrocyte physiology, but also a key disease initiation factor. Additionally, normal 'wear and tear' has been considered a significant risk factor for the development of the disease; however, the fact that the increase in middle zone tissue hydration is the first detectable change, and without any change to the collagen content, this must be resulting from weakening of the collagen network (Stockwell, 1991, Gardner, 1992). These changes vary significantly from the processes taking place during aging, whereby cartilage loses hydration and becomes 'dry'. If the effect of the physio-chemical environment of the chondrocytes on their physiology is considered, it is possible to hypothesize that a group of susceptible chondrocytes, with changes to their physiology, start a sequence of events leading to the production of mechanically compromised matrix and predisposing cartilage to damage and degradation (Hall *et al*., 1996).

However, other factors are also implicated in this intricate disease process. OA is generally regarded as primarily non-inflammatory arthropathy. Nonetheless, elevation of pro-inflammatory cytokines in the cartilage of many OA patients has been detected (Westacott and Sharif, 1996, Fernandes *et al*., 2002) and local inflammation has been observed in animal models of the disease (Goldring, 2000a, Rai *et al*., 2008). Elevated levels of pro-inflammatory cytokines induce the expression and secretion of cartilage-degrading proteases by chondrocytes, leading to the degradation of cartilage ECM (Shlopov *et al*., 2000, Fernandes *et al*., 2002).

Additionally, chondrocyte apoptosis and senescence are processes that can also lead to impaired ECM synthesis (Kim *et al*., 2000, Aigner *et al*., 2004a, Roach *et al*., 2004, Kim and Blanco, 2007). Generally, a variety of chemical stimuli appear to promote chondrocyte apoptosis by elevating pro-inflammatory cytokines, nitric oxide (NO), and Fas ligand (FasL) production and release.

1.2.3.1   Etiology and Epidemiology of OA.

Osteoarthritis, the most prevalent musculoskeletal system disorder throughout the world, is believed to be a consequence of mechanical and biological events that destabilize the normal link of matrix synthesis and degradation within articular cartilage. OA is exceptionally common, particularly among people over 40 years of age (Guccione, 1997). It represents a major cause of morbidity, disability and social isolation, especially where the hip and knee are involved, as this can lead directly to reduced mobility (Ettinger and Afable, 1994). Radiographic changes of joints occur in the majority of people by the age of 65 and are present in more that 80 % of people over 75 years old (Arden and Nevitt, 2006). OA is a disease affecting joint cartilage and the subchondral bone. Its earliest pathologic manifestation is the degradation of cartilage, which when extensive, is visible on radiographs as a narrowing of joint space. Consequent changes in the bone include eburnation of underlying bone, osteophyte formation and sometimes formation of cysts on the subchondral bone (Martel-Pelletier *et al*., 1999). Additionally, there are radiographically visible lateral ostheophytes, before any changes in the joint space can be detected. The disorder leads to significant increase in morbidity, especially in the elderly (Guccione, 1997), whilst osteoarthritis of the knee and hip have the highest social cost and most associated disability (Bergstrom *et al*., 1986).

Knee OA is very common and frequently symptomatic illness, often associated with disability, whereas hip OA is slightly less prevalent with symptoms, which are often more severe and more frequent. Knee osteoarthritis prevalence increases with age (Lawrence *et al*., 1966, Hernborg and Nilsson, 1973, Bergstrom *et al*., 1986, Felson *et al*., 1987) from negligible in those aged 25 – 34% to 20 – 40 % prevalence in people aged >70 years. The prevalence is higher in males than in females up to approximately 45 years old, after which the reverse is true, probably due to the inhibitory effect of estrogen on matrix metalloproteinases (MMPs) production and activation (Claassen *et al*., 2010). Population-based radiographic prevalence England surveys were performed for hip OA and found that radiographic OA increases with age in subjects aged 55-74 years, with 16% of men and 6% of women affected. However, other studies of Caucasian populations in Switzerland and Israel have reported equal rates in

older men and women and lower rates (ranging from 4-7 %) in general as compared to the studies in England (Lawrence and Zinn, 1970, Zinn, 1970, Danielsson *et al*., 1984). It has been found that race plays an important role in the incurrence of OA, with Afro-Caribbean, Chinese and Asian showing much lower rates of hip OA than Caucasian (for a review see: (Allen, 2010, Allen *et al*., 2010).

There are a number of possible causes for the increase of disease prevalence with age including metabolic changes occurring with ageing, which are not parallel to biochemical changes, but may render cartilage more susceptible to fatigue fractures (Claassen *et al*., 2011). However, there are other risk factors that have been found to correlate with the incidence of OA. Population based studies have found an association between obesity and radiographic OA of the hands, feet and knee (Denisov *et al*., 2010), but clinical studies evaluating this relation have not been consistent. Animal studies on rodents have demonstrated that major joint injury causes OA, whereby OA is actually induced by controlled tears of meniscus and cruciate ligament (Troyer, 1982). Such damage is often associated with damage to articular cartilage, the primary lesion of OA and changes in biomechanics, with the latter causing increased shear stress on local areas of articular cartilage. Job occupation can also influence the rate of OA incidence, with repetitive use of particular joints appearing to be a major risk factor for osteoarthritis especially of the hands (McCarthy et al., 1994, Rossignol et al., 2005, Fontana et al., 2007, Ding et al., 2010). This has also been identified within the knee (Dahaghin *et al*., 2009, Reid *et al*., 2010, McWilliams *et al*., 2011) and hip (Rossignol *et al*., 2005, Franklin *et al*., 2010), although the specific physical activity that causes it is not well understood. Other studies have identified other possible risk factors for osteoarthritis, such as diabetes, hypertension, cardiovascular disease, peripheral vascular disease, congestive heart failure, which have also been suggested to play a role in the initiation of osteoarthritis of knees, hips, hands and spines of patients (Felson *et al*., 2000, Solomon, 2001, Marks and Allegrante, 2002b, Marks and Allegrante, 2002a, Singh *et al*., 2002, Bray, 2004, Parada-Turska and Majdan, 2005). There is still, however, the need for new work exploring factors associated with the initiation of osteoarthritis.

1.2.3.2 Molecular mechanisms involved in cartilage degradation.

Often the different forms of arthritis are divided into inflammatory and non-inflammatory, where inflammatory indicates cellular inflammation resulting from the infiltration of various activated leukocytes into the joint and mediating cartilage destruction. Even though osteoarthritis has been usually referred to as a non-inflammatory pathology, due to the lack of infiltrating inflammatory cells in early stages of the disease, there is mounting body of evidence indicating that cartilage degradation is largely the result of chondrocyte-borne inflammation at a molecular level (Goldring, 2000b, Pelletier *et al*., 2001b, Attur *et al*., 2002). Abnormal mechanical forces appear to 'awaken' adult chondrocytes from a state of low metabolic activity and stimulate the production of a host of pro-inflammatory mediators. These include pro-inflammatory cytokines and chemokines, such as IL-1β, IL-6, IL-8, IL-17, IL-18, monocyte chemoatractant protein 1 (MCP1) and reactive oxygen species (ROS; such as superoxide, hydrogen peroxide [H_2O_2] and peroxynitrite). These together with lipid-derived inflammatory stimuli (including prostaglandins and leukotrienes) serve to increase the catabolic activity of chondrocytes (Shlopov *et al*., 2000, Hardy *et al*., 2002, Fernandes *et al*., 2002, Gosset *et al*., 2010). The result is an increase in the release of matrix metalloproteinases (MMPs) and aggrecanases, which can directly degrade the cartilage matrix. It has been proposed that, at least in part, the damage of the cartilage matrix might be an attempt of the chondrocytes to remove and replace the damaged matrix (Shlopov *et al*., 2000, Hardy *et al*., 2002, Fernandes *et al*., 2002, Gosset *et al*., 2010).

1.2.3.2.1 Cytokines.

In addition to age-related and biomechanical changes in the function of chondrocytes, inflammation, and the following deregulation of cytokine synthesis are major contributors to the misbalanced rate of anabolism and catabolism (Fernandes et al., 2002, Goldring and Berenbaum, 2004). Recently, *in vivo* and *in vitro* studies have demonstrated the involvement and the effects of pro-inflammatory cytokines, and particularly IL-1β and TNF-α, in the destruction of articular cartilage during osteoarthritis (Fraser *et al*., 2003, Goldring and Berenbaum, 2004, Kobayashi *et al*., 2005).

IL-1β, also known as catabolin, is a small, 17 kDa, cytokine protein encoded by the *IL1B* gene in humans (Auron *et al*., 1985, March *et al*., 1985). IL-1β, a member of the interleukin 1 cytokine family, is proteolytically activated by caspase 1 and has important functions in the inflammatory response. It is involved in various cellular activities, such as cell proliferation, differentiation and apoptosis (Oppenheim *et al*., 1989, Friedlander *et al*., 1996, Beasley and Cooper, 1999)

TNF-α, or cachexin, is a cytokine implicated in systemic inflammation, with particular relevance to acute phase reaction stimulation. It is a 17kDa protein, encoded by *TNFA* gene, with a primary role in the regulation of immune cells as a potent inducer of inflammation and apoptosis. Two receptors that recognize TNF-α have been identified and termed TNFR1 (TNF receptor type 1; p55/60) and TNFR2 (TNF receptor 2; p75/80), with TNFR1 being expressed by most tissues and TNFR2 being mainly expressed on the cells of the immune system (Locksley *et al*., 2001, Hehlgans and Pfeffer, 2005). Interestingly, the density of TNFR1 has been shown to be significantly greater in chondrocytes isolated from OA lesions compared to those isolated from areas not affected by the disease (Westacott *et al*., 1994), thereby proposing an important role for this cytokine in the progression of OA.

Upon binding to TNFR1, TNF-α, causes a conformation change in the receptors, which leads to the activation of the death domain (DD), enabling the adaptor protein (TNF-α receptor type 1 – associated death domain; TRADD) to bind to DD, and thus initiating a series of phosphorylation events. One group of proteins that mediate these phosphorylation events is the mitogen-activated protein kinase group, or MAPK-group (Garrington and Johnson, 1999), which is a serine-threonine kinase family that consists of the c-Jun N-terminal kinases (ERKs) and the p38 MAPK-activated protein kinases, downstream of pro-inflammatory cytokines, osmotic stress, or apoptotic signals (Lin *et al*., 1995, Aizawa *et al*., 2001). The stimuli first activate MAPK kinase kinases or MAPKKKs, which in turn phosphorylate and activate the MAPKK, kinase that then activates MAPK. MAPKK and MAPK then translocate into the nucleus of the respective cell, where they phosphorylate important transcription factors, including activating protein 1

(AP-1; a member of the c-Jun family) which is especially involved in *MMP13* transcription activation (Lim and Kim, 2011).

TNF-α and IL-1β are strong activators of the NF-κB signalling pathway in chondrocytes. First, TRADD recruits TNF-α receptor associated factor-2 (TRAF2) and RIP (receptor interacting serine-threonine protein kinase). TRAF2 in turn recruits the multi-component protein kinase IKK, enabling RIP-induced activation of it. The inhibitory IκBα is phosphorylated by IKK, which causes its subsequent degradation and ultimately the release and translocation of NF-κB into the nucleus, thereby activating a vast array of pro-inflammatory and catabolic genes (Liu-Bryan and Terkeltaub, 2010). However, it remains controversial whether inflammatory cytokines are primary or secondary regulators of articular cartilage degradation in osteoarthritis. Regardless, it has been shown that physiological loading of cartilage may protect against cartilage degradation by inhibiting the degradation of IKKβ activity in the canonical NF-κB transcriptional activation (Dossumbekova *et al.*, 2007).

TNF-α and IL-1β together with a large number of other cytokines and growth factors trans-activate MMP promoters by convergence of AP-1 and ETS (E twenty six) through activation of p38, JNK and PKC signalling (Figure 1.3) (Iwamoto *et al.*, 1990, Ahmed *et al.*, 2003, Tower *et al.*, 2003, Muddasani *et al.*, 2007, Im *et al.*, 2007, Sampieri *et al.*, 2008, Lim and Kim, 2011). The induction of *MMP13* promoter activity by IL-1β in chondrocytes has been shown to require one or more of the ETS sites and interaction between factors such as RUNX2 and AP-1 (cFos/cJun), which subsequently interact directly with the *MMP13* promoter (Mengshol *et al.,* 2001; Selvamurugan *et al.,* 2004). Interestingly, PGE$_2$, an activator of both AP-1 and Fos-dependent promoters via PKA and PKC signalling pathways has been shown to dose-dependently inhibit the expression of *MMP13*, thereby suggesting a crosstalk between signalling pathways with adjacent transcriptional control elements and opposing effects on *MMP13* transcriptional control (Li *et al.*, 2004)
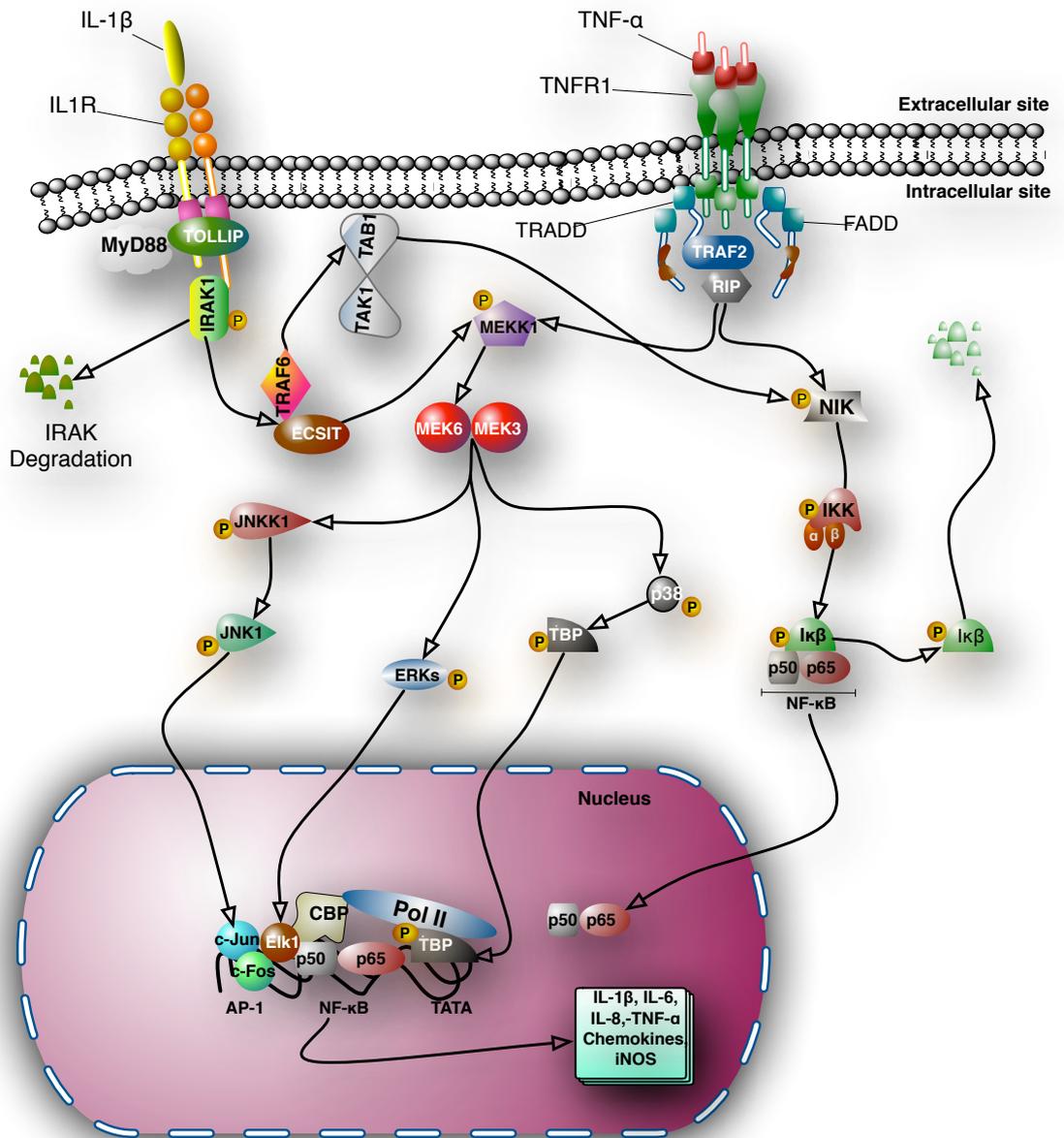
Figure 1.3 IL-1β and TNF-α molecular mechanism of action.

IL1R binds IL-1β but requires the IL1R accessory protein (IL-1RAcP) to transduce a signal. IL-1β binding causes activation of two kinases, IRAK-1 and IRAK-4, associated with the IL1R complex. IRAK-1 (IL1R Associated Kinase) activates and recruits TRAF6 to the IL1R complex. TRAF6 activates two pathways, one leading to NF-κB activation and another leading to c-jun activation. The TRAF associated protein ECSIT leads to c-Jun activation through the MAPK/JNK signalling system. TRAF6 also signals through the TAB1/TAK1 kinases to activate NIK, which phosphorylates IKK therefore triggers the degradation of IκB, and activation of NF-κB (Baud and Karin, 1999). TRAF2 (has been implicated in the activation of two distinct pathways that leads to the activation of Activation Protein-1 (AP-1) via the JNK, MEKK, p38 and together with RIP, NF-κB activation via the NIK (NF-κB Inducing Kinase). TNF-α activates MAPKs: ERK1 and ERK2 (Baud *et al*., 1999)

Other cytokines gaining increasing attention include IL-6, a potent pleiotropic cytokine, expressed by human chondrocytes *in vitro* and *in vivo* (Bender *et al*., 1990, Wang *et al*., 2010a,b), which has been suggested to play an important role in pathogenesis of osteoarthritis. It is thought to elicit both pro-inflammatory and anti-inflammatory effects in cartilage (Bender *et al*., 1990, Goldring, 2000a) through binding to IL-6 receptor (IL-6R), a two-subunit type I cytokine receptor. The IL-6 receptor alpha subunit (IL6Rα) contains the ligand-binding domain and IL-6 signal transducer component (gp130, also called CD130), which is shared by other cytokines from the IL-6 family (IL-11, IL-27, cilliary neurotropic factor [CNTF], cardiotropin [CT-1], CT-like cytokine [CLC], leukaemia inhibitory factor [LIF], oncostatin M; (Kishimoto *et al*., 1995). Binding of IL-6 to its receptor initiates a signal transduction cascade that activates JAK1/STAT3 (Signal Transducer and Activator of Transcription) and extracellular signal-regulated kinases (ERK)-1/2 pathways (Heinrich *et al*., 1998, Heinrich *et al*., 2003, Fischer and Hilfiker-Kleiner, 2007). The activated JAK1 phosphorylates STAT3, which dimerises and translocates into the nucleus, where it activates the transcription of STAT3 response elements (Hirano *et al*., 2000). IL-6 can be directly induced by TNF-α and IL-1β, although IL-6 on its own has been shown to directly augment the expression of *MMP1* and *MMP13* (Shlopov *et al*., 2000)

CXCL8, or IL-8 is a leucocyte chemotactic activating cytokine (chemokine), a member of the CXC chemokine family, encoded by the *IL8* gene, with a primary function in angiogenesis, chemoattraction and activation of neutrophils (Modi *et al*., 1990, Harada *et al*., 1994a, Belperio *et al*., 2000). Various cell types produce IL-8 upon stimulation, as it is one of the major mediators of the inflammatory response (Akahoshi *et al*., 1994, Harada *et al*., 1994 a,b).

Chondrocytes have not only been found to express *IL8,* but the TNF-α-induced activation of NF-κB in chondrocytes also contributes to increased IL-8 protein production (Facchini *et al.,* 2005). In fact, chondrocytes are a rich source of IL-8 and its synthesis is up-regulated in arthritic pathologies and by increased inflammatory cytokines levels (Borzi *et al*., 1999, Pulsatelli *et al.,* 1999). The role of chemokines as second step mediators of local inflammation has gained increasing evidence (Baggiolini, 1998, Luster, 1998), whereby chondrocytes release IL-8 upon stimulation with IL-1β, TNF-α and LPS (Van Damme *et al*.,

1990, Lotz *et al.*, 1992, Recklies and Golds, 1992). Mian and colleagues have demonstrated that IL-8 is involved in NF-κB activation and the over-expression of *MMP2* and *MMP9* and that targeting of this cytokine with anti-IL-8 antibody leads to down-regulation of these proteases (Mian *et al.*, 2003). That suggests that IL-8 can promote increase in matrix metalloproteinases expression, angiogenesis and neutrophil-mediated inflammation in the affected joint (Lotz *et al.*, 1992, Strieter *et al.*, 1992, Hu *et al.*, 1993, Norrby, 1996), leading to further cartilage destruction, and makes it an interesting target for drug intervention.

1.2.3.2.2 Nitric oxide and Cyclooxygenase.

Among other inflammatory mediators found to be involved in the pathological process of OA is nitric oxide (NO). It has been shown that osteoarthritic cartilage secretes large quantities of NO and ROS in addition to high levels of nitrite/nitrates, detected in serum and synovial fluid of arthritis patients (Karan *et al.*, 2003). This process might be caused by the increased expression of inducible nitric oxide synthase, or iNOS (Martel-Pelletier and Pelletier, 2010).

Two iso-forms of cyclooxygenase (COX), COX-1 and COX-2, catalyse the rate-limiting step in the biochemical conversion of arachidonic acid to prostaglandins and thromboxane A_2 (TXA_2) (Smith *et al.*, 1996). COX-1 is a constitutively expressed protein that is thought to produce basal concentrations of prostaglandins and TxA_2 necessary for normal physiologic functions in many tissues. Moreover, COX-dependent production of prostaglandin E_2 (PGE_2) occurs in many tissues through closely regulated and synchronized activities of cytoplasmic phospholipase A_2 ($cPLA_2$), COX, and PGE synthase (PGES, for review, see refs: (Murakami *et al.*, 2000). COX enzymes catalyze the formation of prostaglandin H_2 (PGH_2) from arachidonic acid, followed by the isomerisation of PGH_2 to PGE_2 by PGES. COX-2, PGES, and their major pro-inflammatory product, PGE_2, have been shown to be induced by pro-inflammatory cytokines (Murakami *et al.*, 2000, Vane and Botting, 1998a).

Aberrant expression of COX-2 protein in articular cartilage concurs with significant increase in the levels of PGE_2, an indication of osteoarthritis (Amin *et al.*, 1997), leading to increased chondrocyte apoptosis (Notoya *et al.*, 2000,

Pelletier *et al*., 2001a, Goldring and Berenbaum, 2004). The major prostaglandins synthesized by chondrocytes are PGE_2 and PGD_2. Although some studies have suggested that PGE_2 has anabolic effects at low concentrations (DiBattista *et al*., 1996) and that it can suppress MMP13 and MMP1-mediated catabolism (Tchetina *et al*., 2007), higher concentrations of this prostaglandin, such as those reached during inflammation lead to severe cartilage erosion and chondrocyte apoptosis (Amin *et al*., 1997, Attur *et al*., 2008). Zhu and collagues show that exogenously added PGD_2 diminishes T/C-28a2 chondrocyte cell line viability (Zhu *et al*., 2010).

In addition to regulating metalloproteinases, pro-inflammatory cytokines such as IL-1β and TNF-α have been shown to suppress the expression of *COL2A1* by chondrocytes *in vitro* (Reginato *et al*., 1993, Goldring *et al*., 1994 a,b). In contrast, increased anabolic activity of chondrocytes has been observed in osteoarthritic cartilage, possibly due to the cytokine-induced PGE_2 formation, which up-regulates *COL2A1* transcription (Goldring *et al*., 1994b, Miyamoto *et al*., 2003).

1.2.3.2.3 Anti-inflammatory cytokines and proteins.

In addition to the above mentioned pro-inflammatory cytokines, human chondrocytes have been shown to produce both IL-10 as well as the IL-10 receptor (IL10R) with significantly higher levels in osteoarthritic chondrocytes compared to normal chondrocytes (Iannone *et al*., 2001). IL-10 is a pleiotropic cytokine with important immuno-regulatory functions, whose actions influence activities of many of the cell types in the immune system. IL-10 has potent anti-inflammatory properties, repressing the expression of inflammatory cytokines such as TNF-α, IL-6 and IL-1β by activated cells. IL-10 receptor (IL10R) is a complex of tetramers consisting of two ligand-binding subunits (IL10R1) and two accessory signalling subunits (IL10R2). Upon binding of IL-10 to the extracellular domain of IL10R1, JAK1 and Tyrosine Kinase-2 (TYK2), which are constitutively associated with both IL10R1 and IL10R2, respectively, are activated by phosphorylation (Riley *et al*., 1999). These kinases then phosphorylate specific tyrosine residues on the intracellular domain of IL10R1, which when phosphorylated, serve as temporary docking sites for STAT3. STAT3 is then

phosphorylated by JAK1 and translocates to the nucleus, where it binds with high affinity to SBE (STAT-Binding Elements) in the promoters of various IL-10 responsive genes (Riley *et al*., 1999). IL-10 has also been reported to interfere with the activation of p38/MAPK pathway, thus inhibiting the activation of NF-κB. Direct stimulation of OA chondrocytes with IL-10 has been shown to inhibit the TNF-α-dependent activation of osteoarthritic chondrocytes and therefore to down-regulate the expression of *MMP1* and *MMP13* (Shlopov *et al*., 2000*)*. Interestingly, IL-10 (Lee and Chau, 2002) induces heme oxygenase-1 (HO-1), which is expressed and functionally active in chondrocytes from OA cartilage (Fernandez *et al*., 2003). HO-1 is implicated in the protection against tissue injury and is modulated by cytokines such as IL-1β and TNF- α, the latter of which down-regulate HO-1(Fernandez *et al*., 2003).

HO-1 expression can be strongly induced at transcription level in most tissues by various stress factors including heavy metal, heat shock, ultra violet radiation, endotoxin, hypoxia, hyperoxia, ischemia, hydrogen peroxide, pro-inflammatory cytokines (i.e. IL-1β, TNF-α), NO, cellular glutathione storage depletion and high levels of its substrate heme (Wagener *et al.*, 2003). Contained within the promoter region of HO-1 are the oxidative stress-responsive transcription factor NF-E2-related factor 2 (Nrf2), NF-κB, AP-1, AP-2 and CREB. Alternatively, HO-1 can be negatively regulated by agents such as scavengers of ROS due to their ability to reduce oxidative stress within the body (Lautier *et al.*, 1992). Primarily, HO-1 induction prevents cellular damage and tissue injury by removing excess free heme within the body. In addition, heme acts as a prosthetic group for the activity of inflammatory enzymes including iNOS and COX-2 (Willis, 1999) and is involved in the generation of ROS and RNS that causes cellular stress (Jeney *et al.*, 2002).

1.2.3.2.4   Matrix metalloproteinases in OA.

Proteinases are involved in essential steps of cartilage and bone homeostasis. Normal cartilage ECM is in a state of dynamic equilibrium, with a balance between anabolism and catabolism, brought about by the balance between the proteinases that degrade the cartilage and their inhibitors. It is believed that a disturbance of this balance in favour of catabolism, leads to the pathological

degradation of cartilage ECM observed in osteoarthritis (Kevorkian *et al*., 2004). MMPs are induced by TNF-α, IL-1β, which in combination with IL-6, IL-8, oncostatin M, and other pro-inflammatory mediators, synergistically enhance MMPs production *in vitro*, *ex vivo* and *in vivo* (Shlopov *et al*., 2000, Hui *et al*., 2003, Hall *et al*., 2003, Cawston *et al*., 2003).

MMPs are responsible for the enzymatic cleavage of peptide bonds (Turk, 2006, Lah *et al*., 2006) for the completion of many biological processes, such as digestion, blood coagulation, immune functions, development, apoptosis and processing of precursors needed for collagen synthesis (Dickinson, 2002). Proteases selectively hydrolyse a peptide bond in the polypeptide chains of the target protein and are subdivided into two main groups depending on the location of the preferred peptide bond – endopeptidases and exopeptidases. Exopeptidases are subdivided into aminopeptidases or carboxypeptidases – peptidases that cleave substrate molecules at the amino-terminal or the carboxy-terminal position, respectively (Barrett, 1980, Cawston and Wilson, 2006). Endopeptidases cleave in the middle of the target polypeptide chain and can be subdivided into groups depending on the chemical group involved in the process of catalysis – aspartic-, threonine- and cysteine-proteinases (intracellular, functioning at acid pH), or serine- and metallo- proteinases (extracellular, act at neutral pH; Figure 1.4)(Cawston and Wilson, 2006).

Degradation of the components of ECM is accomplished by a family of more than 26 zinc-containing endopeptidases termed matrix metalloproteinases (MMPs), which are either secreted into the extracellular space, or attached to the plasma membrane of the respective cell (Visse and Nagase, 2003). They share homologous amino acid sequences, contain conserved domains related to their specific substrate preference, and allow recognition of other proteins (Visse and Nagase, 2003). As a family, MMPs can degrade nearly all of the cartilage ECM components, although each individual member is specialized in cleaving specific molecules.
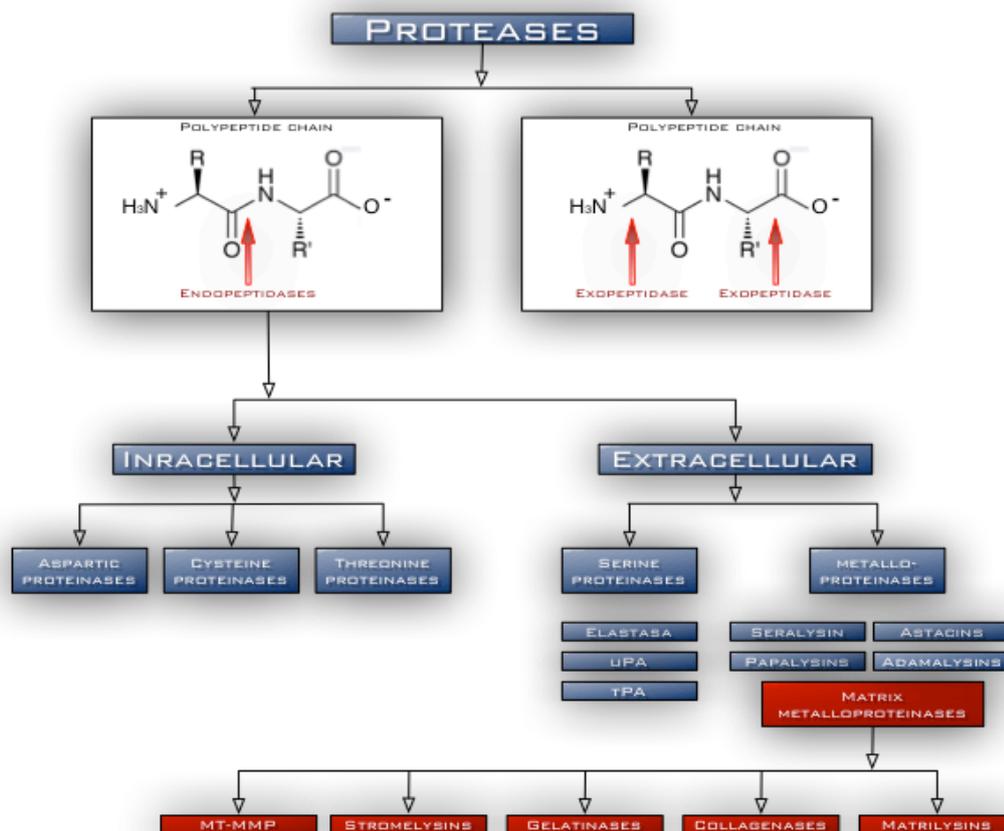
Figure 1.4 Summary of proteases.

In general, MMPs are composed of three distinct domains (Rannou *et al*., 2006): a pre-domain, needed for enzyme maturation and secretion from the cell; pro-domain, required for the maintenance of the enzyme in inactive state; and the catalytic domain, which contains a zinc atom and performs the hydrolysis of the peptide bond. MMPs are categorized into at least five main groups, according to their substrates, cellular localization and primary structure: collagenases, gelatinases, stromelysins and membrane bound (MT)-MMPs (Table 1.1; Figure 1.4). MMPs are tightly controlled at several levels – transcriptional control, pro-enzyme activation, and inhibition of active enzymes by endogenous inhibitors (Chakraborti *et al*., 2003). At transcriptional level, MMPs gene expression is regulated by pro-inflammatory cytokines and growth factors. After binding to their specific receptors, these stimuli activate an intracellular cascade of events, leading to the activation of AP-1 transcription factors and that leads to the up-regulation of MMPs expression (Martel-Pelletier *et al*., 2001).

Table 1.1 Groups of MMPs.

Group	MMPs
Collagenases	MMP1 (collagenase-1)*, MMP8 (collagenase-2), MMP13 (collagenase-3)*, MMP18 (collagenase-4)
Gelatinases	MMP2 (gelatinase A, MMP9 (gelatinase B)
Stromelysins	MMP3 (stromelysin-1)*, MMP10 (stromelysin-2) MMP11 (stromelysin-3)
Matrilysins	MMP7, MMP26
MT-MMPs	MMP14 (MT1-MMP), MMP15 (MT2-MMP), MMP16 (MT3-MMP), MMP17 (MT4-MMP), MMP24 (MT5-MMP), MMP25 (MT6-MMP).

MMPs, matrix metalloproteinases; MT, membrane type.

*Proteases studied in the present study.

Another mechanism of regulation is the secretion of pro-enzymes MMPs. The proteinases are secreted in inactive form, and require the removal of the pro-domain by MT1-, MT2-, MT5-MMP, plasmin, uPA (urokinase) for activation (Chakraborti *et al*., 2003). Upon removal of the pro-domain, MMPs become transiently unstable and undergo conformational change, involving a dissociation of a cysteine residue from the zinc atom in the catalytic domain, thereby exposing the active site and allowing for trans-auto activation of the MMP (Chakraborti *et al*., 2003).

Most cells in the body express MMPs, even though some proteases are associated with a particular cell type. For example the principle substrate for MMP2, or gelatinase A and MMP9, or gelatinase B is the type IV collagen. MMP3, also called stromelysin activates MMP1, or collagenase-1, and is able to cleave a broad range of matrix proteins (Vincenti *et al*., 1996). Both MMP1 and MMP3 are amongst the most ubiquitously expressed collagenases, unlike

MMP13, or collagenase-3, which is expressed primarily by cartilage and bone during development and by the chondrocytes in OA (Borden and Heller, 1997, Mengshol *et al*., 2000, Martel-Pelletier *et al*., 2001, Kevorkian *et al*., 2004).

The only mammalian interstitial proteases that can specifically cut triple-helical interstitial collagens (type and type II) at natural pH are collagenases (Gadher *et al*., 1990, Kevorkian *et al*., 2004), which cleave at a single locus three quarters from the N-terminus, leading to the unwinding of the α-chains. The major collagenases able to perform this enzymatic reaction (MMP1, MMP8 and MMP13) were found elevated in OA cartilage compared to normal tissue (Reboul *et al.,* 1996; Martel-Pelletier *et al.,* 2000). The specific role of MMP8, however, in OA progression remains to be investigated.

IL-1β and TNF-α are thought to be the most important cytokines relevant to osteoarthritis, with increased levels in OA chondrocytes (Tetlow *et al.,* 2001). They are thought to down-regulate anabolic genes such as aggrecan and type II collagen, as well as to directly up-regulate the expression of *MMP1*, *MMP3* and *MMP13* (Shlopov *et al*., 1997, Bau *et al*., 2002). MMP13 specifically hydrolyses type II collagen more rapidly and efficiently than MMP1 and MMP8 (Mitchell *et al*., 1996). Chondrocytes obtained from cartilage adjacent to OA lesions expressed high levels of both *MMP1* and *MMP13 (*Shlopov *et al.,* 1997).

Kevorkian and colleagues conducted the expression profiling of MMPs in cartilage samples obtained from the hips and knees of normal and OA patients (Kevorkian *et al*., 2004). Examination of collagenases showed that *MMP8* expression was undetected thus suggesting that it is unlikely to play a role in cartilage destruction. *MMP9* and *MMP2,* however, are significantly over-expressed in OA cartilage. The inducible MMP9 is thought to have a secondary role, since it contributes to cleaving collagen fibres only after the chains of the triple helix have been already cleaved by the interstitial collagenases (Stetler-Stevenson *et al*., 1997). In contrast MMP2 and MMP14 (MT1-MMP) are both constitutively expressed and minimally regulated.

MMP13 expression is highly increased in OA, consistent with the belief that it is the predominant protease in osteoarthritis (Kevorkian *et al*., 2004). Interestingly, *MMP1* expression decreased in cartilage samples from patients with hip OA, but

was found to be highly increased in osteoarthritic knee cartilage, suggesting that the regulation of this collagenase may differ between joints. A dramatic decrease in *MMP3* in diseased knee cartilage has also been found, and therefore a maintenance function of this protease, which is dysregulated in OA, is suggested (Kevorkian *et al.,* 2004; Bau *et al.,* 2002).

1.2.3.2.5 Chondrocyte apoptosis and OA.

Apoptosis, or programmed cell death, is a normal process during which an orchestrated sequence of events leads to cell death, primarily executed by enzymes called caspases (Kerr *et al*., 1972). Apoptosis is critical not only during development and tissue homeostasis, but also in the pathogenesis of a variety of diseases. Recent studies have revealed that apoptosis is associated with the onset and development of OA (Blanco *et al*., 1998, Kim *et al*., 2000, Sharif *et al*., 2004, Musumeci *et al*., 2011), whereby the proportion of apoptotic cells in OA is greater compared to that in healthy cartilage (Aigner *et al*., 2001). The expression of several caspases, such as caspase-3 and caspase-8, has been found to be increased in human osteoarthritic cartilage and in animal models of the disease (Sharif *et al*., 2004, Robertson *et al*., 2006)

Caspases are a unique family of cysteine-aspartic proteases (Alnemri *et al*., 1996), which are triggered in early stages of apoptosis and are responsible for the activation of most of the events leading to cell death, by cleaving more than a dozen protein kinases, including focal adhesion kinases (FAK), Protein Kinases B & C (PKB & PKC), Raf1 and Lamins. The latter make up the inner lining of the nucleus, and their destruction leads to disintegration of the nuclear lamina, and shrinkage of the nucleus (Kerr *et al.,* 1972)

Apoptosis can be triggered by intrinsic stimuli such as DNA damage, or by extrinsic stimuli such as cytokines. One of the major cytokines, produced by variety of cells in response to infection or multitude of inflammatory stimulants is TNF-α. Activation of its receptor, TNFR1, a member of a family of death receptors is involved in the initiation of apoptosis. It is suggested that the TNFR1 is a preassembled trimer, located on the plasma membrane, the cytoplasmic domain of each subunit of which contains a segment of about 70 amino acids called "death domain" (DD). Death domains of TNF-α receptor specifically self-

associate to induce cell death, by either activating AP-1 and NF-κB (Figure 1.5), or inducing apoptosis. Binding of TNF-α to its receptor causes a conformational change in the death domain, which initiates the recruitment of a number of proteins as indicated in Figure 1.5, two pro-caspases join the protein complex and undergo proteolytic cleavage that yields active caspase-8. Caspase-8 is called an initiator caspase, because it instigates the apoptotic process by activating effector caspases, such as caspase-3, directly involved in carrying out apoptosis (Le *et al.*, 2002).
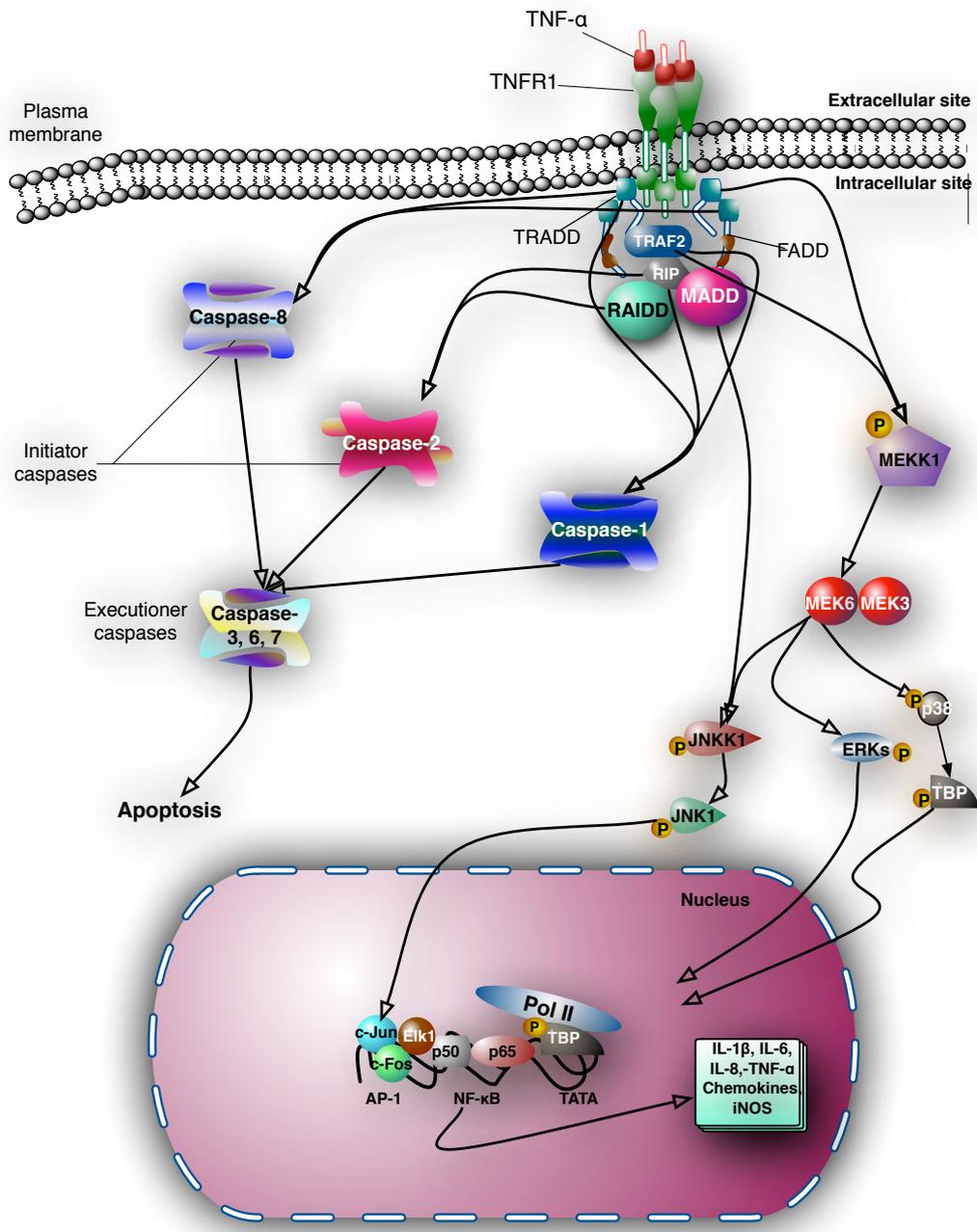
Figure 1.5 Molecular mechanism of caspase-driven programmed cell death.

Initially, TRADD (TNFR-Associated Death Domain) protein, binds to TNFR1. Then, TRADD recruits FADD (Fas-Associated Death Domain), RAIDD (RIP-Associated ICH-1/CED-3-homologous protein with a Death Domain), MADD (MAPK Activating Death Domain) and RIP (Receptor-Interacting Protein). Binding of TRADD and FADD to TNFR1 leads to the recruitment, oligomerization, and activation of Caspase-8 Activated Caspase8 subsequently initiates a proteolytic cascade that includes effector Caspases (Caspases 3, 6, 7) and ultimately induces apoptosis.

1.2.4   Current therapies for the treatment of OA.

A vast array of novel therapies have been developed over the last decade or so for the treatment of arthritic pathologies, especially RA, which now sit alongside the more traditional approaches that include methotrexate, non-steroidal anti-inflammatory drugs (NSAIDs), and glucocorticoids (for review, see: Getting *et al*., 2009). The most widely used therapies for OA are physiotherapy; non-selective COX-1/2 inhibitors such as ibuprofen, naproxen, usually prescribed with protein pump inhibitors such as omeprazole, to alleviate GI side-effects; COX-2 selective inhibitors, or 'coxibs' are another choice of therapy, usually given with low dose aspirin, to prevent CV side-effects, and a proton pump inhibitor. A third choice of therapy is glucocorticoids, prednisone being the drug of choice, administered via intra-articular (i. a.) injection.

1.2.4.1   Non-steroidal anti-inflammatory drugs (NSAIDs).

NSAIDs form an important class of drugs, the therapeutic applications of which have spanned several centuries. The ability of NSAIDs to treat fever and inflammation dates back about 3500 years ago to the time when Hippocrates prescribed an extract from willow bark. In the 17th century, the active ingredient of the bark salicin was discovered, in 1860, the German Kolbe company started producing salicylic acid for the first time and in 1899, Bayer started mass production of Acetylsalicylic acid, or as we know it aspirin (Vane, 2000). The mechanism of action of drugs like aspirin and Indomethacin was revealed by Professor Sir John Vane ~ 40 years ago (Vane, 1971).

The primary effect of NSAIDs is pain relief, but they also exhibit anti-pyretic and anti-inflammatory effects (Vane, 1971) but do not modify the underlying causes of chronic diseases, and these drugs are thus prescribed for symptomatic relief only. The success of the NSAIDs in treating inflammatory disorders such as RA and OA was due to its inhibitory effects on the enzyme cyclooxygenase (COX) (Vane and Botting, 1998b) NSAIDs inhibit prostaglandin synthesis, which can lead to gastro intestinal (GI) toxicity, limiting its use in chronic inflammatory conditions such as OA (Tamblyn *et al*., 1997). Seminal work by Prof. Sir John Vane, Prof. Roderick J. Flower and colleagues led to the identification of two iso-

forms of COX enzyme – COX-1 and COX-2 (Flower and Vane, 1972). The traditional NSAIDs, prescribed for the treatment of joint pain and inflammation, are known to bring about their anti-inflammatory effects by non-selective inhibition of COX activity. However, during inflammation, expression of COX-1 levels do not change, whilst COX-2 levels are significantly up-regulated (Rao and Knaus, 2008), leading to increased production of pro-inflammatory prostaglandins. COX-1 on other hand does not appear to be associated with inflammation, but is highly expressed in the GI tract (Kargman *et al*., 1996), where it is involved in the production of cytoprotective PGE_2 and PGI_2, thereby reducing gastric acid secretion (by the parietal cells in the stomach), up-regulating mucosal blood flow and initiating production of viscous protective mucus. This explains the adverse effect of the traditional NSAIDS (Lazzaroni and Bianchi Porro, 2004). COX-1 isoform is also widely expressed in platelets, where they are responsible for the formation of pro-aggregatory thromboxane A_2 (TxA_2).

These adverse effects led to the development of selective COX-2 inhibitors to bring about a more targeted and specific anti-inflammatory effect (Lazzaroni and Bianchi Porro, 2004). However, post-marketing pharmacovigilance of selective COX-2 inhibitors including celecoxib and rofecoxib (VIOXX), highlighted an ~5 fold increase in myocardial infarction (Mukherjee *et al*., 2001), explained by the effect of the selective COX-2 inhibitors on PGI_2 production. PGI_2 is a vasodilator and a potent inhibitor of platelet aggregation (McAdam *et al*., 1999), which was significantly attenuated by the selective COX-2 inhibitors. Although selective COX-2 inhibition has no effect on TxA_2 production, they tip the natural balance between the TxA_2 and PGI_2 production, thus leading to an increased risk of thrombotic cardiovascular event as demonstrated more recently (Cannon *et al*., 2006). Studies showed that selective inhibition, knock down or mutagenesis of COX-2, or deletion of the receptor for PGI_2, accelerated thrombogenesis and elevated blood pressure in mice, an effect found to be attenuated by COX-1 knock-down (Cheng *et al*., 2006). Discoveries like this led to the withdrawal of the very selective COX-2 inhibitor rofecoxib from the market in September 2004, just 5 years after it was introduced.

1.2.4.2　Glucocorticoids.

Glucocorticoids are endogenously produced hormones implicated in carbohydrate and protein metabolism and exert a potent regulatory effect on inflammation, innate and acquired immune response (Auphan *et al*., 1995). Additionally, they are potent anti-inflammatory drugs inhibiting the synthesis of pro-inflammatory cytokines, interleukins and iNOS, and the production of PGE_2 by inhibiting the transcription of COX-2.

Glucocorticoid receptor is a member of the family of nuclear receptors, which upon binding to their cognate ligands, migrate to the nucleus and act as positive or negative gene transcription regulators (Schacke *et al*., 2004)(Figure 1.6). In the case of positive regulation they trans-activate glucocorticoid response elements (GREs) that are located on the promoter region of the responsive genes (Beato *et al*., 1995, Mangelsdorf *et al*., 1995, Kastner *et al*., 1995). Glucocorticoid receptors are able to negatively regulate transcriptional activation of certain genes in two descriptive mechanisms, one of which is referred to as trans-repression. Trans-repression involves repression of transcription factor AP-1, thus inhibiting its dissociation from the promoter (Konig *et al*., 1992). Another mechanism of action of glucocorticoid receptors is the repression of the NF-κB transcription factor family by physically associating with p65 subunit (Mukaida *et al*., 1994, Ray and Prefontaine, 1994, Caldenhoven *et al*., 1995) In addition, in most cell types excluding endothelial cells, the glucocorticoid receptors trans-activates the IκBα gene transcription, thus leading to prevention of NF-κB translocation in the nucleus (Auphan *et al*., 1995, Scheinman *et al*., 1995, Brostjan *et al*., 1996).

Glucocorticoids are effective anti-inflammatory therapies; however side effects, including suppression of the immune response, healing impairment and gastric ulceration, can limit their use (Schacke *et al.,* 2002). When used as anti-inflammatory agents, glucocorticoids also influence water and electrolyte balance, leading to Cushing's syndrome, easy bruising, and redistribution of the fat in the abdominal region. Additionally, glucocorticoid drugs influence bone density by Ca^{2+} regulation and phosphate metabolism (Guaydier-Souquieres *et al*., 1996). While these adverse effects are not a problem in short-term oral therapy, or more localized application of the drugs (such as i.a. injections or topical ointments), they severely limit the long-term use of glucocorticoids in chronic diseases such as RA and OA.
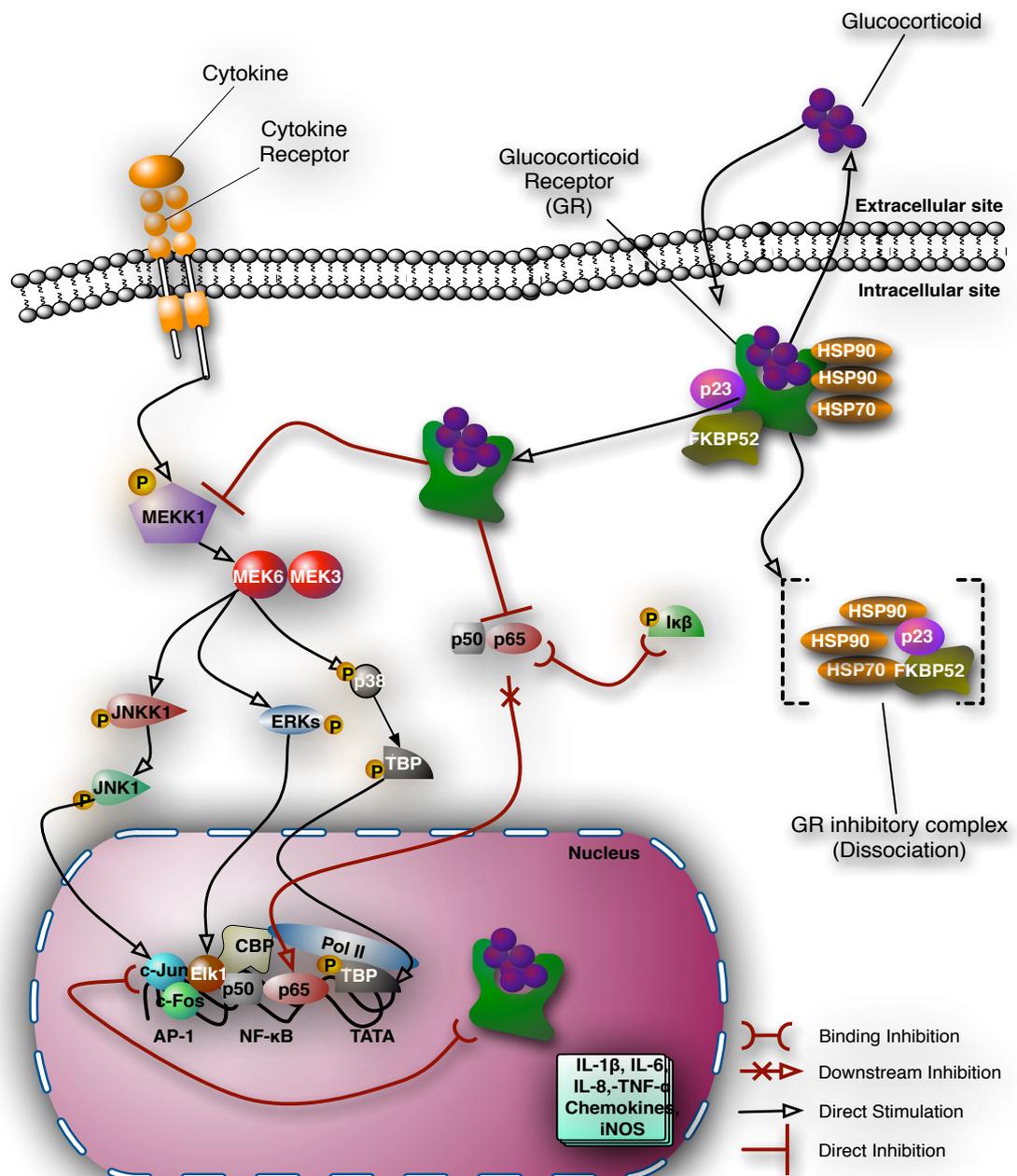
Figure 1.6 Glucocorticoids mechanism of action.

Glucocorticoid receptors are maintained in an inactive oligomeric complex by regulatory proteins such as HSP90 (Heat Shock Protein 90 kD), which after dimerizing bind to HSP70, FKBP52 and p23 phosphoprotein, all of which form the GR inhibitory complex. Upon binding to its glucocorticoid ligand in the cytoplasm, the inhibitory complex dissociates, induces conformational change that exposes the nuclear localization signal of the GR, which then translocates to the nucleus (Pelaia *et al.*, 2003). There, the GR negatively regulate (trans-repress) gene transcription via physical interactions with transcription factors, activated by various pro-inflammatory stimuli (cytokines, etc.) such as the components of AP-1 – c-Jun and c-Fos, and the p65 domain of NF-κB (Schoneveld *et al.*, 2004, Davies *et al.*, 2005). Other mechanisms by which glucocorticoids NF-κB-mediated activation of pro-inflammatory genes are by reducing the levels of phosphorylation of the carboxy-terminal domain of RNA Polymerase II, which is essential for the recruitment of this enzyme to the promoter region and by increasing the transcription and synthesis of IκB, thus causing NF-κB retention into the cytoplasm. The glucocorticoid family are also suggested to negatively regulate the MAPK family (JNK, ERK1, ERK2 and p38), therefore exerting their anti-inflammatory effects (Li *et al.*, 2003).

1.2.4.3   Biologicals.

TNF-α is a primary pro-inflammatory cytokine detected in the synovium and plasma of RA patients and OA cartilage, and is known to be able to activate an array of pro-inflammatory cytokines, including IL-1β, IL-6, IL-8 and MCP-1. Anti-inflammatory therapy with monoclonal antibodies (mAbs) directed against TNF-α has emerged in the 1990's as a major advancement in the treatment of various immune-mediated diseases such as RA, ankylosing spondylitis, psoriatic arthritis and Crohn's disease. A huge boost to anti-inflammatory therapy developments has been the launch of the protein-based injectable etanercept (Enbrel®), and anti-TNF antibodies infliximab (Remicade®), adalimumab (Humira®) (Palladino *et al.*, 2003, Wong *et al.*, 2008), also referred to as 'biologicals'. These anti-TNF monoclonal antibodies exert their beneficial effects by scavenging the pro-inflammatory cytokine TNF-α.

Infliximab is a chimeric monoclonal anti-TNF-α antibody, which binds to soluble and membrane-bound TNF-α, but not to TNF-β, and is able to effectively regulate and mediate inflammatory processes involved in a number of different inflammatory diseases such as RA, Crohns disease, and ulcerative colitis (Bingham, 2008, Haveran *et al.*, 2011). Other anti-cytokine drugs available on the market are etanercept – a TNF receptor covalently linked to the Fc chain of IgG molecule, and anakinra – an IL-1 antagonist. Infliximab, adalimumab and etanercept inhibit the effect of TNF-α by binding to it, although etanercept can additionally bind to lymphotoxin-α. This can be of interest in the treatment of juvenile arthritis, due to lymphotoxin-α production by inflamed tissues (Heiligenhaus *et al.*, 2011, Sevcic *et al.*, 2011).

Even though a beneficial effect has been assigned to these drugs in the clinical management of chronic arthritis and other inflammatory diseases, biologicals lead to a marked increase in opportunistic infections including tuberculosis (TB) with a 3-4-fold higher risk in patients taking infliximab compared to etanercept (Dixon *et al.*, 2010). In part, this could be due to latent TB and so routine screening of individuals prior to commencing therapy is necessary. A recent review by Wallis identified that TNF antibodies pose a greater risk than soluble TNF-α receptor antagonists (Wallis, 2009). A good example is Infliximab, which

when used for prolonged period of time to treat inflammatory disease such as inflammatory bowel disease and Crohn disease causes psoriasis, urinary tract infections, nausea, rashes, decreased white and red cell count and platelet count, vasculitis, and development of lymphoma (Lee *et al*., 2007, Ko *et al*., 2009, Fiorino *et al*., 2009). A number of cytokine antagonists are in development for treatment of RA, including those that target IL-6 (tocilizumab; approved for use in Japan), IL-15, and RANKL, as well as kinase inhibitors, including those that inhibit JAK-3 and SyK (Bingham, 2008). Although some patients respond well to these agents, development of novel therapies is essential should patients become refractory to current therapies.

1.2.4.4   Endogenous novel therapeutics

Annexin 1 is a member of the annexin superfamily of calcium and phospholipid binding proteins (Gerke and Moss, 2002, Perretti and Gavins, 2003). Due to its up-regulation by glucocorticoids, Annexin 1 has been suggested to function as a cellular mediator of the anti-inflammatory effects of glucocorticoids (Roviezzo *et al*., 2002). Cytokines such as IL-1β, TNF-α and IL-6 have been shown to augment Annexin 1 expression (Perretti and Gavins, 2003). A variety of functions have been attributed to this peptide, including inhibition of cell proliferation (Croxtall and Flower, 1992), anti-migratory and anti-inflammatory effects (Flower and Rothwell, 1994, Philip *et al*., 1997, Gerke and Moss, 2002, Perretti *et al*., 2002), cell differentiation regulation, and membrane trafficking (Diakonova *et al*., 1997, Traverso *et al*., 1999). The anti-inflammatory properties of Annexin 1 have been already demonstrated in animal models of RA and myocardial infarction (Perretti *et al*., 1993, Yang *et al.,* 1999, La *et al.,* 2001)

The potential involvement of Annexin 1 in human conditions has been best described in RA, where the endogenous peptide inhibits experimental arthritis in animal models, and upon treatment with anti-annexin-1 antibody, the disease progression exacerbates severely and prevents glucocorticoid drug-mediated anti-inflammatory effects (e.g. dexamethasone) and up-regulates PGE$_2$ and TNF-α production by the synovium (Yang *et al*., 1997). These data suggested that advocating administration of annexin-1 derivatives, or non-glucocorticoid

manipulation of endogenous annexin-1 expression, may prove a beneficial treatment approach in arthritis and other inflammatory diseases.

In concordance with the increasing interest in investigating the properties of endogenous anti-inflammatory mediators, the family of galectins is progressively examined for their immuno-regulatory and anti-inflammatory properties. Galectins (type of lectins, which bind to β-galactosidase), of which 14 mammalian subtypes have been identified, are wildly distributed in animals and possess a variety of functions, including inhibition of chronic inflammation (Dumic *et al*., 2006, Norling *et al*., 2009).

Interest has been directed towards Galectin-1, -3 and -9, as these have been identified as important players in modulation of both acute and chronic inflammatory diseases (Norling *et al*., 2009). Galectin-1 exhibited anti-inflammatory and immunosuppressive properties in a range of models of inflammatory disorders including colitis (Santucci *et al*., 2003), arthritis (Rabinovich *et al*., 1999) and diabetes (Perone *et al*., 2006). Galectin-3 is found in various epithelial cells and cartilage, as well as inflammatory cells such as macrophages, suggesting a role in inflammation (Henderson and Sethi, 2009). Increased expression of Galectin-3 and -9 have been detected in synovium of rheumatoid arthritis patients and to a lesser extent in osteoarthritic synovium (Ohshima *et al*., 2003, Seki *et al*., 2007), whereas Galectin-1 expression is down-regulated in synovium from patients with juvenile idiopathic arthritis (Harjacek *et al*., 2001). Galectin-3 has been implicated in a variety of processes such as heart failure, fibrogenesis, tissue repair, inflammation, cancer and stroke (van Kimmenade *et al*., 2006, Yan *et al*., 2009a, Yan *et al*., 2009b, Liu *et al*., 2009, de Boer *et al*., 2009).

Another class of endogenously produced peptides, with anti-inflammatory and immunosuppressive efficiencies are the melanocortin peptides (including α-MSH) found to be efficacious in experimental models of allergic inflammation (Grabbe *et al*., 1996, Luger *et al*., 2000), chronic inflammation (Ceriani *et al*., 1994), systemic inflammation (Delgado Hernandez *et al*., 1999), inflammatory bowel disease, brain inflammation (Rajora *et al*., 1997a,b) and autoimmune uveoretinitis (Taylor and Namba, 2001). A mechanistic approach has been identified for α-MSH binding to a family of Rhodopsin-like seven-transmembrane

G-protein coupled receptors, whose activation leads to increase in cyclic adenosine monophosphate (cAMP), thereby preventing NF-κB activation via preservation of IκBα protein and resulting in the reduction of production and synthesis of pro-inflammatory cytokines (Catania *et al*., 2004).

1.3 Melanocortin system.

The "melanophore stimulants" were discovered about 90 years ago during surgical ablation experiments by Hogben and Winten (Hogben and Winton, 1922), in which the pituitary gland has been shown to be implicated in the change of skin colour of amphibian. This led to the recognition of pars intermedia as the origin of biological principle. In the 1950s an isolated frog skin assay was developed, which led to the extraction, molecular characterisation and sequence determination of melanocyte-stimulating hormones (MSHs; melanotropins) (Shizume *et al.*, 1954).

Melanocortins are ancient peptides which can be traced back over 700 million years and are derived from the pro-opiomelanocortin (POMC) gene (Heinig *et al.,* 1995). The melanocortin system has been shown to play numerous roles including melanogenesis, energy homeostasis regulation and modulation of inflammatory pathways, obesity, cardiovascular and sexual health (Wikberg *et al.*, 2000). The discovery of anti-inflammatory and immuno-modulatory functions has led to a renewed interest in alpha-melanocyte stimulating hormone (α-MSH), which along with its related peptides and their cognate receptors are responsible for the transmission of these effects and might present potential treatment options for inflammatory diseases (Getting, 2002, Catania *et al*., 2004).

The isolation and sequence determination of adrenocorticotrophic hormone (ACTH), β-lipotropin and γ-lipotropin demonstrated that the sequence for α-MSH was formed following proteolytic cleavage of ACTH (Nakanishi *et al*., 1979). These findings led to the hypothesis that the longer peptides might be serving as a precursor for the shorter proteins (Nakanishi *et al.,* 1979). In 1979 and after deducing the whole amino acid sequence of the POMC peptide from its cDNA, Nakanishi and colleagues reported that it contains an unknown MSH-like peptide sequence, and identified a region bearing homology with α-MSH and β-MSH within the N-terminal fragment in a core heptapeptide (Nakanishi *et al.,* 1979).

Due to the region separation by a dibasic amino acid cleavage sites, they predicted that the intervening peptide will be a secretory product and termed it γ-MSH, due to the high homology to α-MSH and β-MSH. In the last 30 years POMC-derived molecules from many different species were analysed and their genes sequenced. It has been confirmed that POMC gene is expressed in many different tissues and the bioactive peptides, resulting from its post-translational processing, function as not only neuro-peptide regulators in the brain, but also in the periphery (D'Agostino and Diano, 2010).

1.3.1   Pro-opiomelanocortin (POMC) protein.

The POMC gene is 241 amino acids in humans (Takahashi *et al*., 1981), and 209 in mouse (Uhler and Herbert, 1983) and rat (Drouin and Goodman, 1980). POMC is actively transcribed in several tissues, including the hypothalamus, pituitary and periphery including the immune system, lungs, spleen, melanocytes and the gastrointestinal tract (Wikberg *et al.,* 2000). POMC is functionally inert, but undergoes extensive and tissue-specific post-translational modification in order to generate the range of smaller and biologically active peptides mentioned earlier (Rafin-Sanson *et al.,* 2003; Figure 1.7).POMC is comprised of three domains: the N-terminus region, which contains γ-MSH (melanocyte stimulating hormone); the central highly conserved $ACTH_{1-39}$ sequence with α-MSH at its N-terminus; and the C- terminal β-lipotropin, which can be cleaved to generate β-endorphin (Castro and Morrison, 1997). Many hormones and neurotransmitters are synthesized as large pro-hormones, which require cleavage in order to release biologically active fragments. Within the $ACTH_{1-13}$ sequence and the endorphin system, the cleavage sites are usually located between two pairs of basic amino acid residues (-KK-, -RK-, -RR-, -KR-). There are seven members of the pro-hormone convertases (PC) family including PC1/3, PC2, furin/PACE, PACE4, PC4, PC5/6, and PC7/SPC7/LPC/PC8 (von Eggelkraut-Gottanka and Beck-Sickinger, 2004) and the range of processing enzymes expressed in particular tissue, defines the repertoire of POMC-derived products. Thus, PC1 cleaves POMC into $ACTH_{1-39}$ and β-lipotropin, together with low concentrations of β-endorphin, whilst PC2 cleaves POMC into β-endorphin and β-MSH (Figure 1.7), but not ACTH (Benjannet *et al*., 1991).
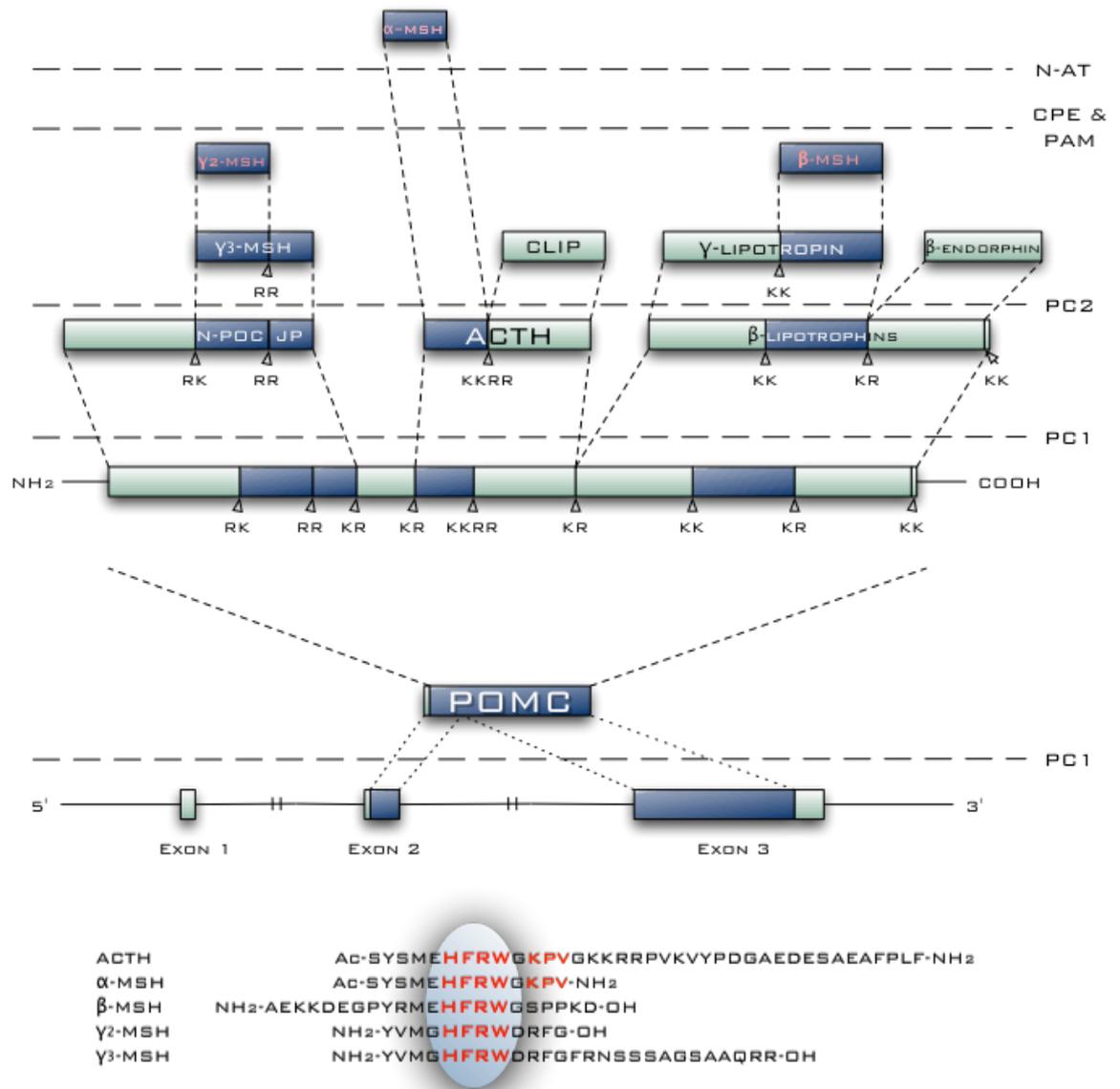
Figure 1.7 Bioactive peptides formed following the post-translational processing of POMC.

POMC in mammals is composed of 3 exons, of which exons 2 and 3 are translated into protein. Prohormone convertases 1 and 2 (PC1/2) break the parent POMC peptide into successively smaller peptides by cleavage at paired dibasic amino acid residues consisting of lysine (K) and/or arginine (R). Cleavage of pro-ACTH by PC1 gives rise to N-POC and joining peptide (JP). The final products are generated in a tissue specific manner by PC2, for example α-MSH and ACTH. They also involve additional enzymatic post-translational modifications, such as in the formation of mature α-MSH - removal of basic amino acid residues by the C-terminal by carboxypeptidases (CPE), amidation by α-amidating mono-oxygenase (PAM) and finally acetylation of α-MSH by n-acetyltransferase (N-AT). The final products include the melanocortins (MSHs and ACTH), β-endorphin and corticotrophin-like intermediate peptide (CLIP). There are intermediate peptides whose biological function remains unclear, such as β-lipotrophin and γ-lipotrophins. All melanocortin peptides contain the common amino acid motif sequence, with ACTH and α-MSH also having in common the tripeptide KPV, which has been shown to antagonize IL-1β effect (highlighted in red).

1.3.2 Melanocortin receptors: Binding and distribution.

Melanocortin peptides exert their biological effects by binding to the smallest family of membrane bound guanine nucleotide-binding protein-coupled receptors (GPCRs). These are composed of seven transmembrane spanning α-helices connected by three intracellular and three extracellular loops and short extracellular N-terminus and short intracellular C-terminus (Catania *et al.,* 2004). All melanocortin receptors possess two highly conserved cysteine residues in their C-terminus that form covalent disulphide bonds and therefore stabilize the receptor structure.

The melanocortin receptor (MC) family consists of five members that have been cloned and termed MC_1 to MC_5. Each receptor is positively coupled to adenylate cyclase; with activation leading to cAMP accumulation following ligation by the common, core tetrapeptide sequence His-Phe-Arg-Trp (HFRW; Wikberg *et al*., 2000; Getting, 2006). Even though α-MSH has equal affinity to MC_3 and MC_4, it has been shown to be somewhat specific to MC_1 (Schioth *et al.,* 1996, 2005; Siegrist and Eberle, 1995). Conversely, β-MSH, γ_3-MSH and ACTH have been shown to have the highest affinity for MC_4 (Schioth *et al*., 1996, Harrold *et al*., 2003), MC_3 (Gantz *et al*., 1993a) and MC_2 (Getting, 2006) respectively.

MCs are expressed in a multitude of tissues (Catania *et al.,* 2004) with varying levels of sequence homology between different receptors. For example, there is 38 % similarity between MC_2 and MC_4 and 60 % between MC_4 and MC_5. In addition to elevations in cAMP, α-MSH can down-regulate NF-κB activation by preventing phosphorylation and degradation of IκB and thus cytokine synthesis (Figure 1.8) (Kelly *et al*., 2006). Increases in intracellular calcium and secondary activation of inositol tri-phosphate have also been proposed as potential mechanisms of action (Wikberg *et al.,* 2000). In addition, PKC and/or PKA recognition sites have been identified on the melanocortin receptors, suggesting that MCs may act as activators of these signaling pathways (Chhajlani and Wikberg, 1992). A number of natural and synthetic melanocortin peptides have been isolated and shown to bind to the five MCs with different efficacies.

1.3.2.1   Melanocortin 1 Receptor (MC$_1$).

MC$_1$, a 317-amino-acid protein cloned in 1992 (Chhajlani and Wikberg, 1992, Mountjoy *et al.*, 1994), is an intronless gene, located on chromosome 16q24.3. It has a multitude of functions including pigmentation, antipyresis and anti-inflammatory, due to its expression peripherally on melanocytes (Chhajlani and Wikberg, 1992; Mountjoy *et al.*, 1992), monocytes (Bhardwaj *et al.*, 1997), neutrophils (Catania *et al.*, 1996), endothelial cells (Hartmeyer *et al.*, 1997), fibroblasts (Hill *et al.*, 2006), mast cells (Adachi *et al.*, 1999), lymphocytes (Neumann Andersen *et al.*, 2001) and macrophages (Star *et al.*, 1995) and within the central nervous system (Xia *et al.*, 1995). A recent publication has unveiled that human chondrocytes express MC$_1$ (Grassel *et al.*, 2009). The receptor is activated by a number of endogenous peptides, with α-MSH being the most active followed by ACTH$_{1-39}$, with β-MSH and γ-MSH causing weak activation (Getting, 2006). Truncated peptides ACTH$_{4-10}$ and ACTH$_{1-10}$ do not activate MC$_1$, suggesting that both the amino and carboxyl-terminal ends of α-MSH (ACTH$_{1-13}$) are important for full biological activation (Tatro, 1996). The relative affinity of melanocortin peptides for MC$_1$ is shown in Table 1.2 bellow. Grässel and colleagues detected not only *MC1* expression in articular chondrocytes in vitro, and in articular cartilage in situ, but they also found transcripts for *MC2*, *MC5* and PCs, and confirmed these receptors were successfully translated into functionally active proteins, which upon activation modulate various pro-inflammatory cytokines, collagens and MMPs (Grässel *et al.,* 2009)

1.3.2.2   Melanocortin 2 Receptor/ACTH receptor (MC$_2$).

MC$_2$ is an intronless gene, located on chromosome 18p11.2 and is translated into a 297-amino-acid long receptor (*homo sapiens*). It is unique among the other melanocortin receptors, as it is activated only by ACTH$_{1-39}$, with no biological efficacy attributed to other melanocortin peptides (Getting, 2006). It has been detected in the adrenal cortex with expression within the zona fasciculata and the zone glomerulosa, the sites responsible for the synthesis and release of glucocorticoids and mineralcorticoids respectively (Mountjoy et al., 1992, Gantz and Fong, 2003) therefore it is essential for steroidogenesis. This receptor was also reported to be expressed in skin (Slominski *et al.*, 1996) and in murine

adipocytes (*mus musculus*) (Boston and Cone, 1996) with a role in lipolysis when activated by ACTH (Boston, 1999). Mutations in this receptor are associated with the rare autosomal recessive disorder Familial Glucocorticoid Deficiency type 1, also known as hereditary unresponsiveness to ACTH (Clark *et al.*, 1993).

1.3.2.3   Melanocortin 3 receptor (MC_3).

MC_3 is an intronless gene located on chromosome 20q13.2-q13.3 and its translation results in a protein, composed of 361 amino acids (*homo sapiens*) and 323 (*mus musculus*). MC_3 is expressed in the periphery and CNS (Gantz *et al.,* 1993a), with $ACTH_{1-39}$, α, β and γ-MSH all equally potent in activating the receptor (Table 1.2).

In contrast to MC_1 the truncated peptides $ACTH_{4-10}$ and $ACTH_{1-10}$ are fully active at MC_3 (Gantz *et al.*, 1993c), suggesting that the core region HFRW is enough for activation. Expression was originally detected in the brain, placenta and the gut (Gantz *et al.,* 1993a) but unlike the MC_1 and MC_2, no expression was detected in melanocytes or adrenal cortex (Gantz *et al.*, 1993a). γ-MSH fails to induce an increase in circulating corticosterone, thereby it has been postulated that activation at MC_3 does not stimulate the hypothalamic-pituitary-adrenal (HPA) axis (Getting *et al.*, 1999). Significant MC_3 expression has been reported within the hypothalamus and the limbic system, as well as the septum, hippocampus, thalamus and the midbrain (Roselli-Rehfuss *et al.*, 1993). This pattern of expression has prompted the search of a central function played by MC_3 which appeared to be energy metabolism (Butler and Cone, 2003). Receptor expression has also been detected in the heart (Guarini *et al.,* 2002, Getting *et al.,* 2004), where it has been demonstrated to mediate myocardial protection (Getting *et al.*, 2004) and in peritoneal (Getting *et al.*, 1999, 2001) and knee joint MØ (Getting *et al.*, 2002) whereby modulating immune response to inflammation (Getting, 2002). The latter is suggested following the observation that activation of MC_3 leads to an initial reduction in pro-inflammatory cytokines and chemokines, followed by the induction of anti-inflammatory mediators at later time points (Lam *et al.*, 2005).

1.3.2.4 Melanocortin 4 receptor (MC$_4$).

Human MC$_4$ is located on chromosome 18q22 and is composed of 332 amino acids with certain similarities to peptides that activate the MC$_1$ (Table 1.2). Expression appears to be restricted to the CNS since detailed studies of various peripheral organs have failed to detect this receptor subtype (Gantz *et al.,* 1993b, Chhajlani, 1996). MC$_4$ is more widely distributed than MC$_3$ within the CNS, being found in the cortex, thalamus, hypothalamus, brain stem and the spinal cord (Mountjoy *et al.,* 1994). The fact that PRO^{12} of ACTH$_{1-39}$, shared by α-MSH, is not present in ACTH$_{4-10}$ or γ-MSH suggests that this amino acid residue could be critical for the binding of MC$_4$ (Gantz *et al.,* 1993b). Physiologically, MC$_4$ activation has been implicated in sexual dysfunction (Gantz and Fong, 2003), energy homeostasis, control of appetite (Butler and Cone, 2003) where the latter are of high interest within the pharmaceutical industry, due to the high demand for treatments of obesity.

1.3.2.5 Melanocortin 5 receptor (MC$_5$).

MC$_5$ was the last MC to be cloned (Gantz *et al.,* 1994) and is located on chromosome 18p11.2, containing 325 amino acids (*homo sapiens* and *mus musculus*), with most potent agonists being α-MSH and ACTH$_{1-39}$. Truncated peptides ACTH$_{1-10}$ and ACTH$_{4-13}$ have been found to provoke full activation of the receptor, although the effectiveness was considerably poorer when compared with α-MSH (Gantz *et al.,* 1994). MC$_5$ is widely expressed in peripheral tissues – liver, lung, thymus, testis, ovary, mammary glands, fat cells, bone marrow, skin, skeletal muscle, stomach and the duodenum (Gantz *et al.,* 1994, Chhajlani, 1996). mRNA expression has also been detected in the olfactory bulb, substantia nigra and striatum of the rat CNS (Griffon *et al.,* 1994). In addition, the expression of MC$_5$ has been reported in B-lymphocytes (Buggy, 1998) and T lymphocytes (Taylor and Namba, 2001), a fact that demonstrates a potential role in immuno-modulation. Other hypotheses on its physiological participation includes: sebaceous secretion, water repulsion, thermal regulation and exocrine gland control (Chen *et al.,* 1997). However, the exact role of MC$_5$ within the body needs to be further investigated.

1.3.3   Melanocortin peptides.

α-MSH corresponds to the first 13 N-terminal amino acid residues of $ACTH_{1-39}$ and is identical in all mammals from which it has been isolated so far (e.g. *Mus musculus*, *Sus scrofa, Macaca mulata, Rattus norvegicus, Ovis aries, Camelus dromedarius, Equus caballus*). The N-terminal serine residue of α-MSH is often N-acetylated and the valine at the C-terminus is almost always covalently linked to a carboxamide group, modifications, rendering α-MSH more potent and stable against exopeptidase activities (Lerner and McGuire, 1964). The structure of β-MSH peptides of different vertebrates is more variable as compared to α-MSH. All β-MSH share 6 constant residues – Tyr^5, His^9-Phe^{10}-Arg^{11}-Trp-^{12} and Pro^{15} and have an acidic iso-electric point (pI), as compared to α-MSH, which has higher pI. Conversely, γ-MSH sequence is not found in POMC precursor molecule of all vertebrate species. It is a dodecapeptide named Lys-γ_1-MSH or just γ-MSH, and in its longer form (22-31 aa) is termed γ_3-MSH. Significant sequence variations of the C-terminal sequence has been identified between species, whilst the N-terminal domain highly conserved (with Tyr^2, His^6-Phe^7-Agr^8-TRP^9 and Phe^{12} being the invariant residues in all species). γ-MSH is processed from γ_3-MSH by cleavage at the dibasic residue pair RR (Figure 1.6), and contains an N-terminal lysine and carboxamide group that has been most likely generated similarly to those of α-MSH.

In addition to these endogenous melanocortin receptor agonists, two naturally occurring antagonists have been confirmed: the Agouti signalling protein (ASP) and agouti-related protein (AGRP). Agouti's gene, which encodes a 131 aa protein containing a signal sequence, controls the relative amounts of eumelanin and phaeomelanin pigments in mammals. Lu and colleagues noticed that Agouti, produced in hair follicles, acts on follicular melanocytes to inhibit α-MSH elicited eumelanin production (Lu *et al*., 1994). What is more, it has been proven that agouti is a high-affinity antagonist to both MC_1 and MC_4 and acts by preventing α-MSH from activating adenylate cyclase (Lu *et al.,* 1994), thus a role in inflammation has been proposed (Catania *et al.,* 2004). It is now wildly known that ASP (the human equivalent of mouse agouti) is broadly distributed, with expressions in the foreskin, liver, kidney, adipose tissue, heart, testis, and ovary

(Wilson *et al*., 1995, Voisey and van Daal, 2002). Contrary, AGRP, is specifically expressed in the CNS – the neuronal cell bodies of the posterior hypothalamus in particular (Catania *et al*., 2004) and has been demonstrated to be an antagonist of MC_3 (Ollmann *et al*., 1997). Additionally, AGRP blocked the anti-inflammatory effects of the selective MC_3 agonist D[TRP]^8-γ-MSH in a murine model of urate crystal peritonitis (Getting *et al*., 2006).

1.3.4   Synthetic melanocortins.

The melanocortin peptides are implicated in the regulation of pigmentation, adrenal function, memory, feeding behaviour, inflammation and more, as described previously (see section 1.3.2). Molecular cloning and sequencing of the melanocortin receptors has boosted systematic studies for investigation of the molecular mechanisms implicated in these functions. Synthetic and non-peptide development have enhanced our understanding of the biological functions of MCs in different tissues. The complexity and the wide expression distributions of the receptors, together with the high sequence homology has impeded the identification of all of their biological effects and has hindered the development of selective agonists and antagonists (Grieco *et al*., 2002). Identification of endogenous and synthetic peptides have been utilised to evaluate the pathophysiological role of the various MCs in different tissues. Extensive investigations led to the identification of the minimum fragment needed for activation of the MCs – HFRW, except MC_2 (Wikberg *et al.,* 2000). A number of peptides and non-peptide compounds have been developed over the last 20 years with varying degrees of selectivity to help elucidate the roles played by each receptor in disease pathologies.

Table 1.2 Melanocortin receptors distribution, ligand specificities and physiological function.

Melanocortin Receptor	Main site of expression	Ligand preference	Main physiological function
MC$_1$	Melanocytes, macrophage, neutrophils, endothelial cells, fibroblasts, chondrocytes, CNS;	α-MSH= β-MSH= ACTH>> γ-MSH	Pigmentation, **anti-pyretic and anti-inflammatory function**;
MC$_2$	Adrenal cortex (zona fasciculata and zone glomerulata), skin;	**ACTH only**	Adrenal steroidogenesis;
MC$_3$	CNS, Placenta, stomach, pancreas, heart peritoneal, knee joint macrophages, chondrocytes;	γ-MSH = α-MSH = β-MSH = ACTH	Energy homeostasis, **anti-inflammatory function**, myocardial protection;
MC$_4$	CNS, spinal cord	α-MSH= β-MSH = ACTH > γ-MSH	Appetite regulation, energy homeostasis, erectile function, pain;
MC$_5$	Lymphocytes, exocrine glands, skin, adrenal gland, adipose tissue, kidney, lymph nodes, liver, skeletal muscle.	α-MSH > β-MSH = ACTH > γ-MSH	Regulation of exocrine gland secretion.

MC$_{1-5}$ – melanocortin receptors; ACTH – adrenocorticotrophic hormone; MSH – melanocortin stimulating hormone; CNS – central nervous system

1.3.4.1 Melanocortin receptor agonists.

[Nle^{4}D-Phe^7]-α-MSH (NDP-α-MSH) was one of the first synthesised analogues of α-MSH to display efficacy in inflammatory models (Sawyer *et al.,* 1980), however, due to its length, the economic reality of bringing this peptide to market led to the development of smaller compounds. Given the short half-life and size of the peptide, smaller fragments were developed, including the potent MC$_{3/4}$ lactam-based heptapeptide super agonist melanotan II (MTII: Ac-Nle-c[Asp-*D*Phe-Lys]-NH$_2$;, EC$_{50}$ = 2.8 nM) (Hadley *et al.*, 1998) shown to be effective in models of murine obesity (Fan *et al.*, 1997) and gouty arthritis (Getting *et al.*, 1999, 2001).

Further identification of the natural agonists of the MC$_3$ led to the discovery that Tryptophan substitution at position 8 into the natural sequence of γ-MSH leads to a selective compound termed D[TRP]^8-γ-MSH (D[TRP]^8-γ-MSH: H-Tyr-Val-Met-Gly-His-Phe-Arg-*D*Trp-Asp-Arg-Phe-Gly-OH; EC$_{50}$= 0.33 nM), which has been recognized to have a 300-fold and 250-fold improvement in selectivity for the MC$_3$ over MC$_4$ and MC$_5$, compared to the naturally occurring γ-MSH (EC$_{50}$ = 5.9 nM; Grieco *et al.*, 2000).

These findings highlighted that those amino acids changes within the polypeptide of α-MSH lead to the development of increasingly selective compounds. The discovery of the synthetic selective MC$_1$ agonists MS05 (Ser-Ser-Ile-Ile-Ser-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH$_3$) and MS09 (Ser-Ser-Ile-Ile-Ser-His-*D*Phe-Arg-Trp-Gly-Lys-Pro-Val-NH$_2$) occurred following screening of peptide phage display library (Martignoni *et al.*, 1997) with MS09 being more potent, but less selective than MS05 (Wikberg *et al.*, 2000). These compounds potently down-regulate the expression and secretion of selectin (E-selectin), vascular cell adhesion molecule (VCAM), and intracellular endothelial cell adhesion molecules (ICAM) in human dermal vascular endothelial cells treated with TNF-α. In addition, they down-regulate IL-1β induced NF-κB activation in these cells (Brzoska *et al.*, 1999). The octapeptide 154N-5 (Met-Phe-Arg-*D*Trp-Phe-Lys-Pro-Val-NH$_2$), which showed a high degree of HTB-14, HEK293, Jurkat, TNP-1 cell-lines, primary human cells and animal model (C3H/HEN mice) of TNF-α secretion (Ignar *et al.*, 2003).

1.3.4.2   Melanocortin antagonists.

Development of SHU9119 is one of the major breakthroughs in melanocortin pharmacology. Substitution of the bulky aromatic amino acid DNal(2') into position 7 of ACTH$_{1-39}$ led to the discovery of the antagonist at MC$_3$ and MC$_4$ with the structure Ac-Nle-c[Asp-His-DNal(2')-Arg-Trp-Lys]-NH2 (Hruby *et al.*, 1995). SHU9119 has allowed many groups to dissect the role of MC$_3$ in diseases ranging from arthritis to cardiovascular pathologies, and also in models of obesity as it has been shown to be an antagonist at MC$_{3/4}$ in murine model of obesity (Fan *et al.,* 1997; Butler *et al.,* 2000) and models of acute inflammation (Hartmayer *et al.,* 1997; Neumann-Andersen *et al.,* 2001; Hill *et al.,* 2006; Adachi *et al.,* 1999; Getting *et al.,* 1999, 2001). Other substitutions into positions 6 to 15 of ACTH$_{1-39}$ have yielded the MC4 antagonist HS014 (Schioth *et al*., 1999), which blocked cAMP induced by α-MSH in MC$_4$-transfected COS-1 cells. The selective cyclic MC$_4$ antagonist HS024 (Kask *et al*., 1998) has an enhanced 10-fold potency compared to HS014 (Schioth *et al*, 1999) and has been demonstrated to stimulate food intake in rats (Kask *et al*., 1998). In order to investigate further the role of His6 in the core tetrapeptide of the melanocortin peptides, Grieco and colleagues designed, synthesized and characterized new cyclic lactam ligands with several modifications and the most important variant was the substitution of the Histidine residue at position 6 in SHU9119 with a Pro and Hyp (Grieco *et al.,* 2002). This led to the discovery of the full agonists at MC$_5$ – PG901 (Ac-Nle-c[Asp-Pro-DNal(2')-Arg-Trp]-Lys-NH$_2$, EC$_{50}$ = 0.072 nM), approx. 40 times more active than the superagonist MTII (EC$_{50}$ = 2.8 nM), and PG911 (Ac-Nle-c[Asp-Hyp-DNal(2')-Arg-Trp]-Lys-NH$_2$, EC$_{50}$= 0.031 nM), which was synthesized by placing a Hyp residude at position 6). The His-Hyp substitution at position 6 of SHU9119 caused a remarkable agonistic activity of PG911 at MC$_5$. In addition, both peptides showed noteworthy antagonistic activities on MC$_3$ and MC$_4$, compared to SHU9119 (Grieco *et al.,* 2002).

1.3.5 Melanocortin peptides molecular mechanism of action.

The main intracellular signalling mechanism of stimulated melanocortin receptors is cAMP production via MC/Gs coupled to adenylate cyclase (Wikberg *et al.,* 2000). However, the peptides have been shown to not only elicit cAMP increase, but also stimulate p38 pathway, extracellular signalling kinases (ERK; p44/42) (Mandrika *et al.*, 2001), PKC and NF-κB transcription factors AP-1 and NF-κB as outlined in Figure 1.8 (Konda *et al.*, 1994, Kalden *et al.*, 1999, Ichiyama *et al.*, 1999). The binding of melanocortins to its corresponding receptor is thought to activate protein kinase A (PKA), which has four main effects. First, PKA activation induces the phosphorylation of the cAMP-responsive-element-binding protein (CREB), which, owing to its high affinity for the co-activator CREB-binding protein (CBP), prevents the association of CBP with p65 (which is a key component of (NF-κB). Second, activated PKA inhibits IκB kinase (IKK), which stabilizes the IκB inhibitor and prevents nuclear translocation of p65. Third, PKA activation inhibits MAPK/ERK kinase kinase 1 (MEKK1) phosphorylation and activation, and the subsequent activation of p38 and TATA-binding protein (TBP). TBP need to be phosphorylated in order to be able to bind to the TATA box and to form an active trans-activating complex with CBP and NF-κB (Gonzalez-Rey *et al.*, 2007). A reduction in the amounts of nuclear p65, CBP and phosphorylated TBP inhibits the formation of the conformationally active trans-activating complex that is required for the transcription of most cytokine and chemokine genes. Fourth, inhibition of MEKK1 by PKA subsequently deactivates Jun kinase (JNK) and c-Jun phosphorylation. The composition of the activator protein 1 (AP1) complex changes from the transcriptionally active c-Jun–c-Jun, to the transcriptionally inactive c-Jun–c-Fos or CREB (Gonzalez-Ray *et al.*, 2007).

The final consequence is that the transcriptional machinery, which is perfectly assembled to the gene promoters of several inflammatory mediators (examples shown in red box) after the signalling of TNF-α through TNFR1, is significantly disrupted by treatment with melanocortin peptides. In addition to these effects of melanocortin peptides there is another mechanism of action that has been proposed. In human MC_3 transfected HELA cells, activation of cAMP-dependent PKA in the presence of α-MSH was shown to exert an inhibitory effect on the IP3-mediated increase in intracellular Ca_2^+ concentration (Konda *et al.,* 1994).
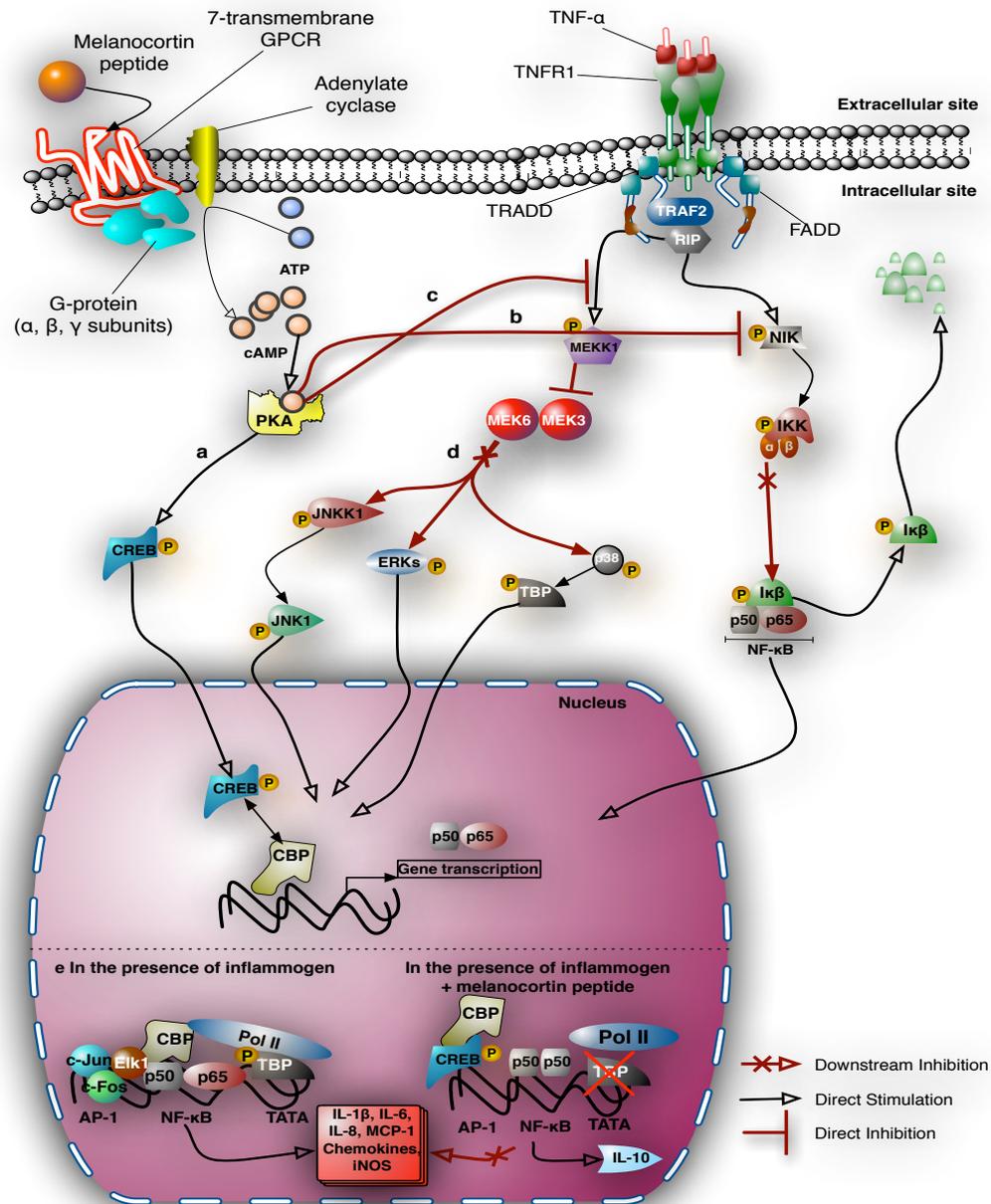
Figure 1.8 Molecular mechanism and transcription factors involved in the anti-inflammatory effects of melanocortin peptides.

The binding of melanocortins to its corresponding receptor increases cAMP and is thought to activate protein kinase A (PKA), which has four main effects. a) First, PKA activation induces the phosphorylation of the cAMP-responsive-element-binding protein (CREB) prevents the association of CBP with p65. b) The activated PKA hinders IκB kinase (IKK), which stabilizes the IκB inhibitor and stops nuclear translocation of p65. c) PKA activation prevents MAPK/ERK kinase kinase 1 (MEKK1) phosphorylation and activation, and activation of p38 and TATA-binding protein (TBP). Non-phosphorylated TBP is unable to bind to the TATA box and to form dynamic trans-activating complex with CBP and NF-κB. A reduction in the amounts of nuclear p65, CBP and phosphorylated TBP inhibits the formation of the conformationally active trans-activating complex that is required for the transcription of most cytokine and chemokine genes. d) Fourth, inhibition of MEKK1 by PKA subsequently deactivates JUN kinase (JNK) and cJUN phosphorylation. The composition of the activator protein 1 (AP1) complex changes from the transcriptionally active cJun–cJun, to the transcriptionally inactive JunB–cFos or CREB. e) The final consequence is that the transcriptional machinery is significantly disrupted by treatment with melanocortin peptides (Gonzalez-Ray *et al.*, 2007).

77

1.4   Role of melanocortin peptides in arthritic pathologies.

1.4.1   Osteoarthritis.

Current treatment regimes for OA largely focus on exercise, surgery for joint replacement, and the treatment of symptoms, e.g., relieving pain with analgesics, rather than treating the underlying causes of the disease (Ettinger and Afable, 1994, Chen *et al*., 2008, Franklin *et al*., 2010)

Only a handful of studies so far have evaluated the therapeutic potential of ACTH and melanocortin peptides in OA. This may seem somewhat surprising at first glance given the number of studies suggesting therapeutic potential of these peptides in both pre-clinical models and human patient samples from RA and gouty arthritis patients. However, this could stem from the fact that whilst inflammation is considered causal to both RA and gouty arthritis, it is not generally accepted to be a major contributor to the development of OA.

Catania and colleagues evaluated the presence of systemic and synovial α-MSH in patients with OA and RA to evaluate whether changes in levels correlated with disease progression. α-MSH was detected in the synovial fluid of both OA and RA patients, with levels that were much lower in OA than RA (Catania *et al.,* 1994). Of interest, however, was the observation that synovial levels of α-MSH were higher than plasma, suggesting a local production of this peptide (Catania *et al*., 1994). This could indicate that activation of resident cells within the joints cause the release of α-MSH, which in turn switches off disease progression in an auto or paracrine fashion. Again, given that inflammation is only considered to be a component of OA rather than a causal factor, this could explain why much lower levels of α-MSH are found in OA rather than RA patients. One potential mechanism for the articular cartilage degradation observed in OA may be due to TNF-α induced expression of MMPs, which can be down-regulated by α-MSH. In the human chondrosarcoma cell line HTB-94 (SW1353), α-MSH has been shown to down-regulate TNF-α induced expression of *MMP13* mRNA and protein (Yoon *et al*., 2008). Studies with the pharmacological inhibitor SB203580, a p38 MAPK inhibitor, showed that α-MSH inhibited *MMP13* by modulating p38 MAPK phosphorylation and subsequent activation of NF-κB (Yoon *et al*., 2008).

Although α-MSH has been shown to decrease TNF-α induced MMP expression, the parent hormone ACTH can cause terminal differentiation of chondrocytes and subsequent cartilage degeneration (Evans *et al.*, 2004). Rodent chondrocytes and chondrocyte cell-lines express MC_3, matrix deposition was concentration dependently elevated in the presence of ACTH. These data highlight that the melanocortin system promotes chondrocyte phenotype development and their differentiation into mature chondrocytes, leading to an elevation in intracellular free calcium (Evans *et al.*, 2005).

1.4.2   Rheumatoid arthritis.

RA is a complex pathology affecting many systems outside of the joints, with 40 % of RA deaths due to cardiovascular disease. Therapeutic intervention in RA is a leading cause of complications, for example NSAIDs may cause elevated blood pressure (Getting *et al.,* 2009) and glucocorticoids increase cardiovascular problems by accelerating the rate of pathologies such as arterial thickening and narrowing (Getting *et al.,* 2009). Methotrexate on the other hand may also promote heart disease by increasing levels of homocysteine. As a result of these complications and the fact that NSAIDs are contraindicated in the elderly due to high possibility of kidney failure, the progress of novel endogenous therapeutics is indispensable.

The role of melanocortin peptides in rheumatoid arthritis has not been fully ascertained yet. Catania et al. detected increased concentration of α-MSH in the synovial fluid of patient suffering from RA, juvenile arthritis, but not OA, that were greater than that detected in the plasma (Catania *et al.,* 1994; Grässel *et al.,* 2009) suggesting local production of the peptide, i.e. that the anti-cytokine molecule α-MSH is produced within a site of inflammation. This increase in α-MSH within synovial fluid also suggests the possibility of an endogenous anti-inflammatory loop maintaining a homeostatic balance within the joint and controlling the host inflammatory response. Joint concentrations of α-MSH in these patients were directly proportional to the degree of inflammation, whilst systemic (plasma) levels remained at physiological concentrations (Catania *et al.,* 1994).

Utilising the adjuvant-induced arthritis model, α-MSH was shown to modulate weight loss, arthritic score, joint damage, and swelling, characteristic features of the disease. This effect on preventing weight loss was in contrast to that observed by the glucocorticoid prednisolone, which caused significant weight loss in these animals (Ceriani *et al.,* 1994). The inflamed joint is characterised not only by infiltrating leukocytes, but also by activated resident bone/cartilage cells, such as osteoclasts and chondrocytes. Some evidence exists to suggest melanocortin receptor expression on these cells (Yoon *et al.,* 2008). In situ hybridization shows that all MCs are expressed on chondrocytes in the mouse femoral bone and mRNA signals for all MCs, except MC_1, were detected on primary rat osteoclasts, which also expressed the POMC gene (Zhong *et al*., 2005), thereby suggesting the possibility that POMC peptides generated by these cells could act in an autocrine/paracrine manner through their corresponding MCs. α-MSH has been shown to inhibit TNF-α-induced *MMP13* by modulating p38 and NF-κB signaling in human chondrosarcoma cell-line HTB-94 (Yoon *et al.,* 2008). These findings indicate that α-MSH may be beneficial in arthritic conditions.

1.4.3   Gouty arthritis.

$ACTH_{1-39}$ was first shown to have clinical efficacy in gout in the 1950s, although no mechanism of action was described for its effects (Gutman and Yu, 1950). Long-term administration of $ACTH_{1-39}$ causes a number of side effects, including suppression of the HPA axis, and thus its use was superseded by other therapies. Current treatments for gout focus largely on the use of colchicines, allopurinol and NSAIDs, which cause a number of side effects, including peptic ulceration, gastric problems, and are poorly tolerated in the elderly and patients with renal insufficiency (Ritter *et al*., 1994), a potential reason why there is a renewed interest in the use of $ACTH_{1-39}$. Another reason is the fact that ACTH demonstrates a greater efficacy than conventional glucocorticoids in controlling gout (Ritter *et al*., 1994). The authors suggested the existence of another mechanism of action other than the well-characterised stimulation of ACTH/MC_2 expressed on the adrenal gland (Ritter *et al*., 1994). Therefore, anti-inflammatory fragments of $ACTH_{1-39}$ that do not activate the HPA axis have been sought in the

hope that they may have a better safety profile. Utilising murine models of gout, it has been demonstrated that smaller fragments of the POMC gene, including the peptides α-MSH, β-MSH, and $ACTH_{4\text{-}10}$, could reduce urate crystal–induced neutrophil migration, and pro-inflammatory cytokine and chemokine release (Getting *et al.,* 1999). Of specific interest are the anti-inflammatory effects of these peptides, which occur in a corticosterone-independent manner and thus there is no reflex stimulation of the HPA axis.

These findings mirror those observed for $ACTH_{4\text{-}10}$ in rat skin inflammation, where it was shown to inhibit prostaglandin generation and oedema formation (Gecse *et al*., 1980). The biological efficacy of these peptides was shown to occur via MC_3 expression on peritoneal macrophages with subsequent elevations in cAMP. Further confirmation of the role of MC_3 in mediating the anti-inflammatory effects of these peptides was highlighted when pre-treatment of mice with the $MC_{3/4}$ antagonist SHU9119 led to an attenuation of their efficacy. Based on these findings, a hypothesis was proposed that MC_3 could be a novel therapeutic target for modulating the anti-inflammatory effects of the melanocortins, at least in this model of monosodium urate crystal deposition (Getting *et al.,* 1999). Further studies promote this hypothesis with natural and synthetic agonists of the MC_3, γ_2-MSH (Gecse *et al*., 1980) and MTII (Fan *et al*., 1997) respectively, attenuating inflammation both *in vivo* and *in vitro* (Getting *et al.,* 2001). In a rodent model developed to mimic certain aspects of the human pathology, urate crystals were injected into the knee joint, which led to migration of neutrophils preceded by the release of IL-1β and IL-6. Local and systemic administration of the parent hormone $ACTH_{1\text{-}39}$ resulted in significant inhibition of all parameters associated with joint inflammation (swelling, arthritic score, neutrophil migration) (Getting *et al.,* 2002).

1.4.4   Role of melanocortins in other inflammatory pathologies.

In addition to the anti-inflammatory properties discussed above, melanocortins have been shown in various *in vitro* and *in vivo* experiments to possess modulatory roles in inflammation. *In vitro*, α-MSH can reduce the T-lymphocyte co-stimulatory molecule CD86 in LPS stimulated monocytes (Bhardwaj *et al.,* 1997), decrease LPS-induced TNF-α production in whole blood (Catania *et al.,*

2000) and THP-1 cells (Taherzadeh *et al.*, 1999) and inhibit NO synergistically elicited by LPS and IFN-γ in a MØ cell-line (Star *et al.,* 1995, Mandrika *et al.,* 2001). Supporting data were also shown *in vivo* in models of endotoxemia (Delgado Hernandez *et al.*, 1999), inflammatory bowel diseases (Rajora et al., 1997b) and experimental heart transplant (Gatti *et al.*, 2002), where melanocortin peptide treatment caused the reduction of NO and TNF-α.

Interestingly, α-MSH has been shown to modulate the elevation, production and release of the anti-inflammatory cytokine IL-10 in human peripheral blood mononuclear cells, thus promoting further the inhibition of pro-inflammatory cytokines induced by MØ (Bhardwaj *et al.*, 1996). The importance of α-MSH-induced IL-10 in mediating anti-inflammatory responses was further substantiated *in vivo*, where the suppressive effect of α-MSH on airway inflammation is no longer observed in IL-10 knockout mice (Raap *et al.*, 2003).

A remarkable facet of melanocortin biology is their ability to regulate peripheral acute inflammation following central administration. It was shown in brain ventricle injection that pre-treatment with α-MSH and ACTH$_{11-13}$ reduced ear and hind paw oedema induced by local injection of IL-1, IL-8, leukotriene B$_4$ and platelet-activating factor (Ceriani *et al.,* 1994, Macaluso *et al.,* 1994). When spinal cord trans-section was performed, these anti-oedema effects caused by central α-MSH injection was abolished, indicating that intact descending inhibitory neural pathways are needed for this effect (Macaluso *et al.*, 1994).

1.5   Aims of the study and hypothesis.

The aims of this thesis were to examine the expression and functionality of melanocortin receptor MC_1 and MC_3 in human C-20/A4 cell-line and primary bovine articular chondrocytes. Another aim was to ascertain their anti-inflammatory, anti-apoptotic and chondroprotective effect on pro-inflammatory cytokine production following stimulation of *in vitro* cultures (C-20/A4 cell-line and primary articular chondrocytes) with $TNF\alpha$/ mechanical impact of chondrocytes *in situ* and to investigate their effect on the production of anti-inflammatory proteins.

- To investigate the response of chondrocytes to inflammatory stimuli and the effect on pro-inflammatory cytokine (IL-1β IL-6, IL-8 and MCP-1), degradive matrix metalloproteinase (*MMP1*, *MMP3* and *MMP13*) and cartilage ECM matrix component (collagen type I and type II) production.

- To study the effect of classical anti-inflammatory drugs glucocorticoids (dexamethasone) and NSAIDs (indomethacin) on expression and release of IL-6, IL-8 and MMPs from activated chondrocytes.

- To ascertain melanocortin receptor expression, production and functionality in chondrocytes and determine whether melanocortin peptides can inhibit pro-inflammatory cytokines, and/or MMP production and induce anti-inflammatory proteins in cell-line and primary chondrocytes.

- To evaluate the protective properties of melanocortin peptides against TNF-α-induced apoptosis and caspases activity.

- To investigate the response of chondrocytes to lowered extracellular osmolarity, *e.g.* pro-inflammatory cytokines and degradive enzymes and the effect of melanocortin peptides on these parameters.

- To characterise the cytoprotective and anti-inflammatory effect of melanocortin peptides on mechanically impacted articular chondrocytes *in situ.*

Chapter 2
Materials and Methods

2.1 Materials and suppliers.

2.1.1 Chemical and reagents.

Table 2.1. Chemicals and Biochemicals used for tissue culture.

Name	Supplier	Catalogue No.
Ascorbic acid	Sigma-Aldrich Company Ltd., Poole, UK	A4403
Foetal Bovine Serum (FBS)	Invitrogen, Paisley, UK	16170078
GIBCO® DMEM + Glutamax™-1	Invitrogen, Paisley, UK	31966
Pen/Strep Solution (+5000 U/ml Penicillin, +5000 µg/ml Streptomycin)	Sigma-Aldrich Company Ltd., Poole, UK	P0781
Sodium Chloride	Fisher Scientific, Leicestershire, UK	S13160/65
Trypsin-EDTA	Invitrogen, Paisley, UK	25300

Table 2.2. Chemicals and biochemicals used in protein extractions, SDS-PAGE and Western blots.

Name	Supplier	Catalogue No.
Acryl Amide	Bio-Rad Laboratories Ltd., Hertfordshire, UK.	161-0100
Ammonium Persulfate	Bio-Rad Laboratories Ltd., Hertfordshire, UK	161-0700
β-mercaptoethanol	Sigma-Aldrich Company Ltd., Poole, UK	P0781
Bio-Rad Protein Assay (Bradford solution)	Bio-Rad Laboratories Ltd., Hertfordshire, UK	04693159001
Bis-acrylamide	Bio-Rad Laboratories Ltd., Hertfordshire, UK.	161-0154
Coomassie© brilliant blue R250	Sigma-Aldrich Company Ltd., Poole, UK	25300
Dithiotreitol (DTT)	Fisher Scientific, Leicestershire, UK	BP172-25
Glycine, aminoacetic acid	Fisher Scientific, Leicestershire, UK	BPE381
Glycerol	Sigma-Aldrich Company Ltd., Poole, UK	49779
Methanol	Sigma-Aldrich Company Ltd., Poole, UK	32213
N,N,N',N'-Tetramethylethylenediamine (TEMED)	Sigma-Aldrich Company Ltd., Poole, UK	T9281
NuPAGE® MOPS SDS Running Buffer 20x	Invitrogen, Paisley, UK	NP0001
NuPAGE® Transfer Buffer 20x	Invitrogen, Paisley, UK	NP0006
NuPAGE® Novex 10% Bis-Tris Gels	Invitrogen, Paisley, UK	NP0301BOX

NuPAGE® Novex LDS Sample Buffer 4x	Invitrogen, Paisley, UK	NP0007
Ponceau S Staining Solution	Sigma-Aldrich Company Ltd., Poole, UK	P3504
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich Company Ltd., Poole, UK	A4403
Trizma® Base	Sigma-Aldrich Company Ltd., Poole, UK	T1503
Trizma® hydrochloride (Tris-HCl)	Sigma-Aldrich Company Ltd., Poole, UK	T3253

Table 2.3 Reagents used in ELISAs.

Name	Supplier	Catalogue No.
3, 3',5,5' –Tetramethylbenzidine tablets	Sigma-Aldrich Company Ltd., Poole, UK	T5525
Bovine albumin serum (BSA; fraction V, minimum 98%)	Sigma-Aldrich Company Ltd., Poole, UK	A2153
Dimethyl Sulfoxide (DMSO)	Fisher Scientific, Leicestershire, UK	D4121
Hydrogen Peroxide (30%)	Fisher Scientific, Leicestershire, UK	H1550
Phosphate Buffer Saline (PBS) tablets	Oxoid LPD, Hertfordshire	BR0014G
Phosphate Citrate Buffer	Sigma-Aldrich Company Ltd., Poole, UK	P4809
Sodium acetate	Fisher Scientific, Leicestershire, UK	S210-500
Tween-20	Fisher Scientific, Leicestershire, UK	BPE337

Table 2.4 Primers used in this study.

Name	Supplier
β-actin forward and reverse	Eurofins MWG Operon, London, UK
Collagen I forward and reverse	Eurofins MWG Operon, London, UK
Collagen II forward and reverse	Eurofins MWG Operon, London, UK
IL-6 forward and reverse	Eurofins MWG Operon, London, UK
IL-8 forward and reverse	Eurofins MWG Operon, London, UK
MMP1 forward and reverse	Eurofins MWG Operon, London, UK
MMP3 forward and reverse	Eurofins MWG Operon, London, UK
MMP13 Forward and reverse	Eurofins MWG Operon, London, UK

	Applied Biosystems UK, Cheshire, UK
MC$_1$ forward & reverse primers	Applied Biosystems UK, Cheshire, UK
MC$_3$ forward & reverse primers	Applied Biosystems UK, Cheshire, UK
MC$_5$ forward & reverse primers	Applied Biosystems UK, Cheshire, UK

Table 2.5. Antibodies used in this study.

Name	Supplier	Cat. No.
α-Tubulin Mouse mAb (clone B-5-1-2)	Sigma-Aldrich Company Ltd., Poole, UK	T5168
Cleaved Caspase-3 (Asp175) (5P1) Rabbit mAb	Cell Signalling Technology Inc., UK	9664
Goat anti-rabbit horseradish peroxidase (HRP)-conjugated IgG	Dako Cytomation Ltd., Cambridgeshire, UK.	P0448
Goat anti-mouse horseradish peroxidase (HRP) conjugated IgG	Dako Cytomation Ltd., Cambridgeshire, UK.	P0447
Heme oxygenase(HO)-1 rabbit pAb	Bioquote Ltd. (supplier for Stressgen Biotechnologies), York, UK.	SPC-112C

Table 2.6 Commercial Kits used in this study.

Commercial Kits	Supplier	Cat. No.
cAMP Biotrak™ EIA system	GE Healthcare UK Ltd., Buckinghamshire, UK	RPN2251
Caspase-Glo® 3/7	Promega UK Ltd., UK	TB323
DuoSet® ELISA Development systems	R&D Systems® Europe Ltd., Abingdon, UK	N/A
GoTaq Green Mastermix 2x	Promega UK Ltd., Southampton, UK	M7122
ImProm–II™ Reverse Transcription System	Promega UK Ltd., Southampton,UK	A3800
NucleoSpin® RNA/Protein	GE Healthcare UK Ltd., Buckinghamshire, UK	740933.50
Parameter™ PGE$_2$ Assay	R&D Systems® Europe Ltd., Abingdon, UK.	KGE004B

Table 2.7. Additional materials and chemicals used in this study.

Materials	Company	Cat. No.
α-MSH; ACTH$_{1-13}$	Sigma–Aldrich Inc. Poole, UK	M4135
3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT)	Promega UK Ltd., Southampton, UK	M2128
3-isobutyl-1-methylxanthine (IBMX)	Sigma–Aldrich Inc. Poole, UK	I7018
Agarose	Sigma–Aldrich Inc. Poole, UK	A9539
Calcein-AM	Anaspec Inc., Freemont, USA	89201
Dexamethasone	Sigma–Aldrich Inc. Poole, UK	D4902
DirectLoad™ 50 bp DNA Step Ladder	Sigma–Aldrich Inc. Poole, UK	D3812
D[TRP]^8-γ-MSH	Kind gift by Prof. Paolo Grieco, University of Naples	N/A
Ethanol, 99.9%	Fisher Scientific, Leicestershire, UK	AC61510-0020
Ethidium bromide (EtBr$_2$)	Invitrogen, Paisley, UK	15585-011
Forskolin	Sigma–Aldrich Inc. Poole, UK	F6886
FUJI Medical X–Ray Film	FUJIFILM EUROPE GmbH, Düsseldorf, Germany	50469
Full–range Rainbow™ Molecular Weight Marker	GE Healthcare UK Ltd., Buckinghamshire, UK	RPN800
Hybond™–C Extra Nitrocellulose membrane	Amersham Biosciences UK Ltd. Buck, UK	RPN303E
Hydrochloric acid (HCl)	VWR International (supplier for BDH), Leicestershire, UK	30024.290
Indomethacin	Sigma–Aldrich Inc. Poole, UK	I7378
Interleukin(IL)-6	Sigma–Aldrich Inc.	SRP4145

	Poole, UK	
KODAK GBX Developer/Replenisher	Sigma–Aldrich Inc. Poole, UK	P7042–1GA
KODAK GBX Fixer/Replenisher	Sigma–Aldrich Inc. Poole, UK	P7168–1GA
Lipopolysaccharide (LPS; 0111:B4)	Sigma–Aldrich Inc. Poole, UK	L4391
Pierce® ECL Western Blotting Substrate	Thermo Scientific, Surrey, UK	32209
Propidium Iodide (PI)	Invitrogen, Paisley, UK	P1304MP
RNase inhibitor	Promega UK Ltd., Southampton, UK	N2611
RQ1 RNase-free DNase	Promega UK Ltd., Southampton, UK	TB518
SHU9119	Phoenix Pharmaceuticals, Karlsruhe, Germany	043-24
Sodium nitrite	Sigma–Aldrich Inc. Poole, UK	S5506
Triton X-100	Fisher Scientific, Leicestershire, UK	BPE151
Trypan Blue solution (0.4%)	Sigma–Aldrich Inc. Poole, UK	T8154
Zymosan Type A	Sigma–Aldrich Inc. Poole, UK	Z4250

2.1.2   Software.

Table 2.8. Software used in this study.

Software	Company
Image J	Bitplane AG, Zurich, Switzerland
Imaris 7.1.1	Imaris
Microsoft Office 2008	Microsoft, USA
GraphPad Prism	GraphPad Software, San Francisco, USA
OmniGraffle Pro 5.02	Washington, USA
Leica TCS SP1/SP2	Leica Microsystems (UK) Ltd., Milton Keynes UK
EndNote X2	Adept Scientific, Herts, UK

2.2   Methods.

2.2.1   Cell line storage and resuscitation.

C-20/A4 is a human chondrocytic cell line, derived from juvenile costal chondrocytes (Finger *et al*., 2004). C20/A4 chondrocytes were stored in liquid nitrogen in 1.0 ml aliquots of 1.0 x 10^6 cells/ml in Dulbecco Modified Eagle Medium (DMEM) supplemented with 50.0 % FCS and 20% DMSO. Prior to cell culture, cells were rapidly thawed at 37ºC in a water bath and pre-warmed complemented medium (DMEM + Glutamax supplemented with 50 U/ml Penicillin, 50 µg/ml Streptomycin and 10% FCS) added to dilute the toxicity of the DMSO. The resulting cell suspension was centrifuged at 2000 rpm for 10 min and supernatant discarded, thus removing DMSO. The cell pellet was then resuspended in complete medium, transferred into 25 cm^2 flasks and incubated in humidified incubator with 95% air : 5% CO_2 to allow cell proliferation.

2.2.2   Cell line culture and maintenance.

Cell culture media was replaced at 4-day intervals whereby media were removed by aspiration and replaced by fresh complemented DMEM. At 80 % confluence, chondrocytes were subcultured by lifting the cells from their substrate using 0.05 % Trypsin-EDTA and centrifuging of detached adherent cells at 2000 rpm for 10 min. The resulting cell pellet was resuspended and seeded in new culture flasks (1.0 x 10^3 cells/cm^2) for continuous culture or in various (6-, 24-, or 96-) well plates (1.0 x 10^5 cell/cm^2) as appropriate for experimentation. Cells were incubated at 37 ºC in a humidified atmosphere supplied with 95% air : 5% CO_2. Cell line passage numbers used in all experiments were between 1 and 10.

2.2.3   Cartilage removal and chondrocyte isolation.

Fresh metacarpal or metatarsal phalangeal joints of 18 – 21 month old female animals were obtained with permission from the local abattoir and were dissected under aseptic conditions on the day of slaughter. Full depth articular cartilage, excluding subchondral bone, was removed carefully from the load bearing surfaces of the metacarpal phalangeal joints and cartilage explants cultured in DMEM (serum-free; 280 mOsm/kg:H_2O; 37ºC, 95% air :5% CO_2) until required

(used within 96h). Osmolarity was measured using Vapro™ vapour pressure osmometer and accurate to ± 5 mOsm (Kerrigan and Hall, 2008).

For chondrocyte isolation, cartilage explants were incubated for ~18h at 37ºC with 0.8 mg/ml Collagenase in DMEM 380 mOsm (Bush and Hall, 2001). The osmolarity of the DMEM was raised by addition of 3.14 mg/ml filter-sterilized NaCl (using 2.0 µm filter) to limit cell swelling upon chondrocytes released from the matrix (Hall *et al.,* 1996b). Digest material was passed through a tea strainer, and 20.0 μm nylon filters (Falcon) to remove undigested material and any large cell clumps. Chondrocytes were washed 3x by centrifugation (8 min, 20º C, 600g) in DMEM (380 mOsm/kg:H_2O). For stimulation of freshly isolated bovine chondrocytes (P_0 – passage 0), chondrocytes were seeded at 1.0 x 10^5 cells/cm^2 in 25 cm^2 plastic flasks in the same serum-free 380 mOsm DMEM for 2 – 24 h to allow for cell attachment.

For long-term culture of primary bovine chondrocytes, cells were isolated as described above and following the third wash, chondrocytes were resuspended in 'complemented' DMEM (380 mOsm/kg:H_2O; 10% FCS, 50 μg/ml ascorbic acid, 50 U/ml Penicillin, 50 μg/ml Streptomycin; pH 7.4; Kerrigan *et al*., 2008). Media was replaced when required and chondrocytes were subcultured up to passage 3 (P_3; 21 days). Briefly, supplemented media was removed and chondrocytes were lifted from the plastic using Trypsin-EDTA 0.05%. The suspension was diluted 1:2.5 in DMEM (380 mOsm) prior to washing by centrifugation (3x). Subsequently, cells were seeded as described above in plastic flasks with various sizes (depending on the experimental needs).

2.2.4   Application of single impact to bovine articular cartilage explants.

A vertical drop tower was used to deliver single blunt impact to individual cartilage explants under aseptic conditions. The impacting weight and the base were made of hard nylon with smoothly polished surface as previously described (Bush *et al.,* 2005). The individual cartilage explants were carefully positioned with the synovial surface uppermost, and exposed to single blunt impact with a weight of 137 g dropped from a height of 10 cm, equivalent to 1.14 N, 6.47 kPa (assuming linear acceleration). Multiple impacts occurred rarely, and these

explants were discarded. The cartilage pieces were then removed from the drop tower and returned to the 10 cm^2 dishes for 6 h when the chondrocytes were incubated for 30 min with Calcein-AM and visualised under confocal laser scanning microscopy (CLSM). Human knee forces are approximately 12.0 kPa, significantly higher than the ones used here (Matthews *et al*., 1977), but it has to be noted that the articular cartilage explant did not have the support of the underlining bone, or the protection of the synovial fluid available *in vivo*. Therefore, the impact tower was used for injuring the cartilage and not as a model of forces found *in vivo*.

Explants of bovine articular cartilage were incubated for 30 min with or without melanocortin peptides α-MSH and D[TRP]^8-γ-MSH (3.0 µg/ml) prior to single impact and then incubated for 6 h. Explants were then incubated with the fluorescent indicators Calcein-AM (5.0 μM) and Propidium Iodide (1.0 μM) permitting the measurement of cell viability using CLSM.

Cell viability of *in situ* chondrocytes was investigated by CLSM and cytokine release (IL-1β, IL-6, IL-8, MCP-1) from cartilage explants by ELISA. Tissue was viewed by placing the cartilage piece in a new dry dish and viewing perpendicularly to the articular (synovial) surface. A small amount of cyanoacrylate glue was applied to a small corner of the explant, at a distant point of that to be viewed and PBS was added.

2.2.5   Confocal Microscopy.

2.2.5.1   Confocal Microscopy Imaging.

Images were taken using an upright Leica TCS NT CLSM (Leica Microsystems, Milton Keynes, UK) and Leica Software. The following parameters were adopted for the acquisition of the images (Kerrigan and Hall, 2005).

- Grey resolution of 8 bit
- Pinhole: 1.00 airy unit, matched to the diameter of objective used
- Beam splitter 488/543/633
- Laser speed 400 Hz
- Dipping lens HCX APO L U-V-1 63.0x 90 W

During the incubation of cartilage explants with Calcein-AM and PI, the molecules diffused through the matrix into the cells.

Calcein-AM is non-fluorescent when outside the cell. It is cell membrane permeable and once in the cell, the molecule undergoes enzymatic cleavage by intracellular esterase's, a process, which releases the fluorophore – Calcein. It is comparatively membrane impermeable and therefore is trapped within the cells. After excitation of the dye with a 488 nm argon laser (emission at 525 nm), Calcein fluoresced, providing useful indication of cytoplasmic space in the living cells. Propidium Iodide (PI) is membrane impermeable and only when bound to nucleic acids, it fluoresced red (excitation at 525 – 535 nm and emission = 650 – 670 nm), therefore indicating the presence of dead cells. When cartilage was impacted, it was possible to observe cell death, as dying cells lost their green fluorescence rapidly as it leaked out of cell membranes upon impact. Instead, they fluoresced red, as the PI entered the cell through the leaky membrane and bound to the nucleic acids.

Chondrocytes in cartilage explants were viewed by an upright Leica TCS SP2 CLSM (Leica Microsystems, Milton Keynes, UK) with low power magnification of x10 air objective lenses for cell viability measurements. Laser power and detector sensitivity were adjusted to provide optimal image quality, preventing dye

bleaching (Bush and Hall, 2001a,b). Scanning speed was 0.6 Hz with double frame integration, double line averaging for 1024x1024 pixel image.

2.2.5.2   Image analysis.

Confocal microscopy collects a series of planar images comprised of voxels (3D pixels). Each voxel contains information about its relative position and intensity. To be able to correctly portray a cell, it was essential to ascertain the correct boundaries of each individual cell. Images of chondrocytes loaded with Calcein-AM showed strong homogenous fluorescence that differed tremendously from the emission of the surrounding matrix/DMEM media, which shows negligible fluorescence under the conditions used for this thesis.

In order to determine cell viability prior to and post impact, CLSM data were imported into and analysed using Imaris 7.1.1 Spots feature. Based on the average diameter of viable cells (green) and "dead" cells (red), in the cartilage explant, spot analysis was used to calculate the total number of cells and the number of viable cells within the area of observation. For spots analysis, the optimum threshold for Calcein-AM and PI was calculated by selecting a smaller area with a known number of cells and different threshold values from 0 – 100% were applied in increments of 10 %. Using the resulted linear regression formula, the correct cell number was determined with a threshold percentage of 20 % for Calcein-AM-stained cells (viable cells) and 60 % for PI-stained cells (dead cells) as shown on Figure 2.2.
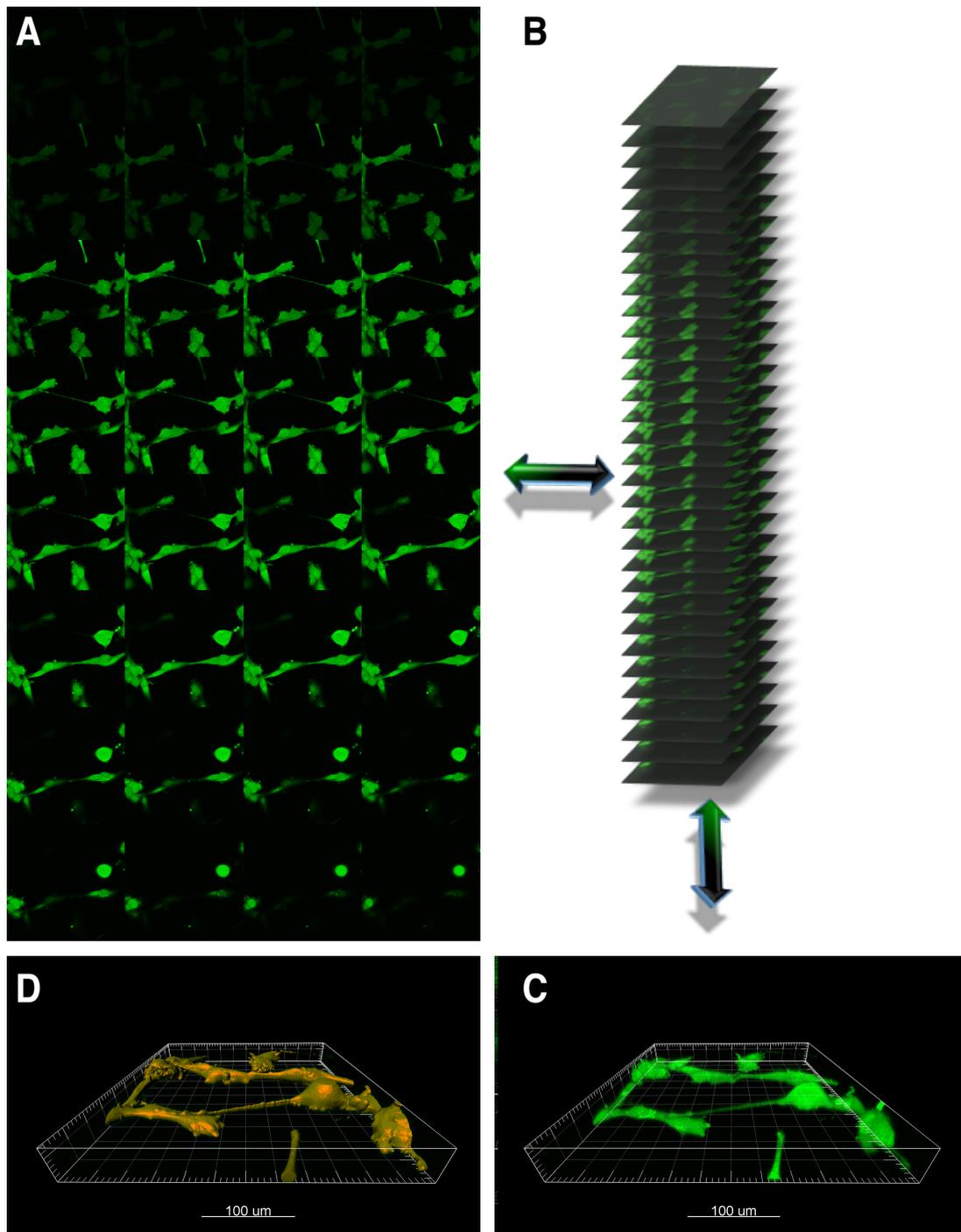
Figure 2.1 Schematic representation of 3D image reconstitution using Imaris 7.1.1. A series of images were taken using upright Leica CLSM along the z-axis of chondrocytes adherent to the surface of a plastic 10 cm^2 culture dish (A). The images were imported in Imaris 7.1.1 and reconstituted (B) to give a 3D view of the observed cells (volume objects; C). Using the Surfaces visualization feature of the software, the correct cell boundaries above the background cut-off were identified by a computer-generated representation of a specified grey value range in the data set. It creates an artificial solid object (D) in order to visualize the range of interest of the volume object.
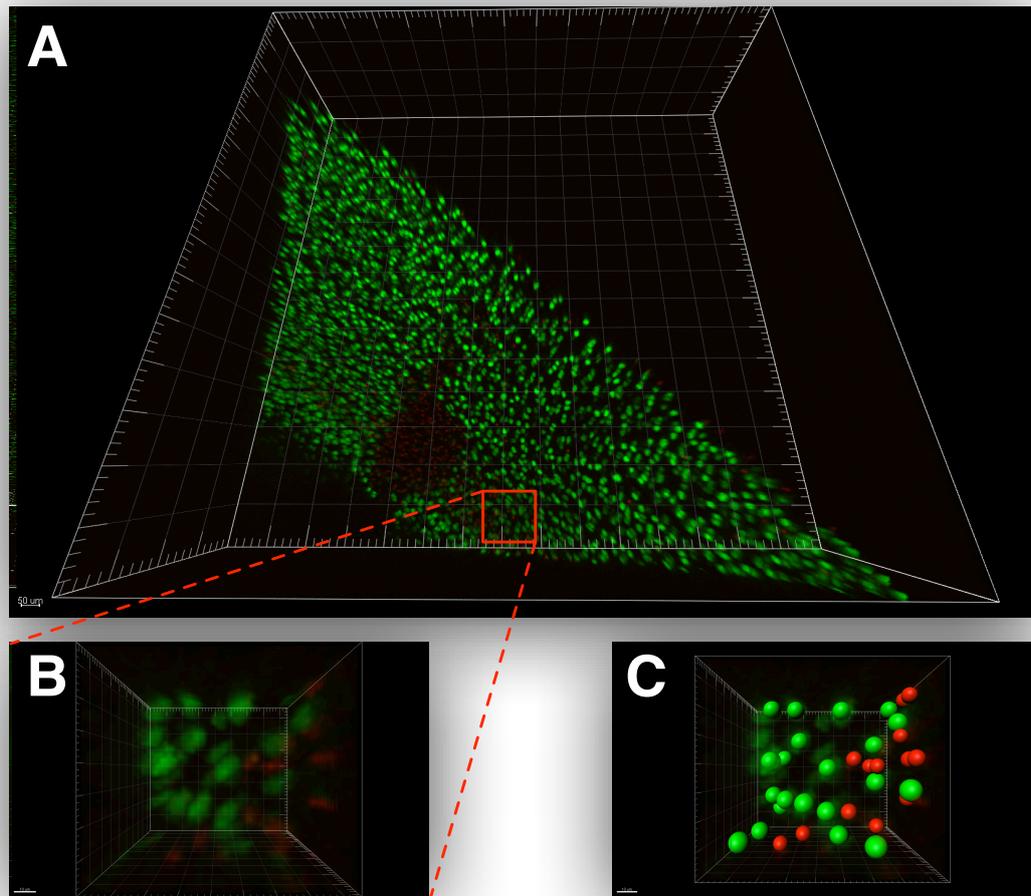
Figure 2.2 Optimisation of spot analysis.

Cartilage explants were stained with Calcein-AM (5.0 µM) and Propidium Iodide (1.0 µM) and imaged under CLSM. An area of visually countable cells (Panel B) was cropped from the 3D CLSM image (Panel A) and spot analysis was applied to the cropped section (Panel C) for both the viable (green) and non-viable (red) cells by altering the baseline threshold in 10 % increments from 0-100 %. Actual cell number was used to accurately choose the optimal threshold percentage for Calcein-AM and PI.

2.2.6　Cell-line and primary bovine chondrocyte stimulation.

2.2.6.1　*In vitro* cell stimulation with various inflammogens.

Primary bovine chondrocytes P_o and C-20/A4 chondrocytes seeded in 96-well plates were stimulated with TNF-α (20.0 – 80.0 pg/ml), LPS (0.1 – 10.0 µg/ml), MSU crystals (0.1 – 10.0 µg/ml), H_2O_2 (0.01% - 1.0%). Both control and stimulated chondrocytes were incubated for various time periods, outlined in figure legends, in humidified incubator at 37ºC, 95% : 5% air : CO_2. Cells were analysed for MTT reduction to assay cell viability and media collected for Griess nitrite accumulation assay and cytokine ELISAs (for detailed protocols, see: sections 2.3.4; 2.3.1 and 2.3.2 respectively).

2.2.6.2　Chondrocyte drug treatment.

2.2.6.2.1　*In vitro* melanocortin agonist treatment of human C-20/A4 and primary P0 bovine articular chondrocytes.

C-20/A4 cell line or primary bovine P_0 chondrocytes were seeded in 6- 24- or 96-well plates and incubated for 24h at 37ºC in humidified incubator at 37ºC, 95% : 5% air : CO_2 to allow cell adhesion to the culture substrate. Culture medium was then replaced with serum-free DMEM medium and chondrocytes incubated in different conditions for various time points (outlined in the respective figure legends). Chondrocytes were treated with a panel of melanocortin peptides – α-MSH, D[TRP][8]-γ-MSH, PG901, PG911, or SHU9119 in a concentration range (0.1 – 30.0 µg/ml) for 2 – 24 h in serum-free DMEM ± TNF-α (60.0 pg/ml) for C-20/A4 cells or for 6 h only (P_0 bovine cells). The cells in the 24-well plates and 96-well plates were analysed for MTT and cell-culture media were collected for cytokine ELISA experiments.

In separate experiments, cells were treated with α-MSH, D[TRP][8]-γ-MSH (3.0 µg/ml) or SHU9119 (10.0 µg/ml) ± TNF-α (60.0 pg/ml) for 6 h. For protein expression and RNA extraction, cells were removed from the 6-well plates with a cell scraper and the respective experiment was performed as described in individual figure legends. The cells in the 24-well plates and 96-well plates were

analysed by MTT assay for cell viability, and cell-culture media were collected for cytokine ELISA experiments.

Alternatively, cells were treated with α-MSH, D[TRP]^8-γ-MSH (3.0 µg/ml) ± SHU9119 (10.0 µg/ml) + TNF-α (60.0 pg/ml) for 6 h and the media from 24-well plates were used for cytokine ELISA measurements. Cells plated in 6-well plates were used for RNA extraction.

2.2.6.2.2 *In vitro* dexamethasone and Indomethacin treatment of human C-20/A4 chondrocytes.

To investigate the effect of classical anti-inflammatory drugs in this system, two widely used drugs were chosen dexamethasone (glucocorticoid) and Indomethacin (NSAID). Human C-20/A4 chondrocytes were pre-treated for 30 min with 1.0 µM of either dexamethasone or indomethacin and then stimulated with TNF-α (60.0 pg/ml) for 0 – 24 h. Total RNA was extracted and reverse transcribed into cDNA. Conventional PCR was employed to investigate the transcription levels of *IL6* and *IL8* at time point 6 h. From the same experimental set-up, cell-free supernatants were collected at time points 0, 2, 4, 6 and 24 h and analysed for IL-6 and IL-8 protein release via commercially available ELISAs.

2.2.6.2.3 *In situ* cartilage explants treatment with melanocortin peptides and dexamethasone.

Cartilage explants were obtained as explained earlier (Section 2.2.3) and treated with PBS, dexamethasone (1.0 µM) or melanocortin peptides – α-MSH/D[TRP]^8-γ-MSH (3.0 µg/ml) for 6 h prior to confocal microscopy for evaluation of their effect on chondrocyte viability. From the same experimental set-up, cell free supernatants were collected to evaluate basal release of IL-1β, IL-6 and IL-8 and to compare that to the effect of the abovementioned agents.

Alternatively, in order to evaluate and compare the protective properties of the melanocortin peptides and steroids on mechanically injured cartilage explants, the cartilage pieces were incubated for 30 min with PBS (control), dexamethasone (1.0 µM) or melanocortin peptides – α-MSH/D[TRP]^8-γ-MSH (3.0

μg/ml) for 30 mins prior to cartilage impact (using drop tower as explained in section 2.2.4). The cartilage explants were then returned to their vials immediately, whereby they were incubated for additional 6 h at 37°C prior to confocal microscopy visualization for identification of the effect of the treatments on cell death and changes in chondrocyte morphology. Cell-free supernatants were collected at that time-point in order to evaluate: 1.) the effect of mechanical trauma on pro-inflammatory cytokine production by the injured chondrocytes, and 2.) the therapeutic, anti-inflammatory effect of dexamethasone, α-MSH and D[TRP]^8-γ-MSH on chondrocytes following mechanical trauma.

2.2.7   mRNA and protein analysis

2.2.7.1   RNA purification and quantification.

Total RNA was extracted from primary and cell-line chondrocytes using the commercially available NucleoSpin® RNA II Kit. Cell culture medium was aspirated and the cells washed with PBS, prior to trypsinizing them using 0.05 % Trypsin. Cell suspension was centrifuged at 3000 rpm for 5 min and subsequently lysed by incubation of the cell pellet with 350 μl buffer RA1 (containing 3.5 μl β-ME), which contains large amounts of chaotropic ions, therefore immediately inactivating RNases, creating appropriate binding conditions favoring adsorption of RNA to the silica membrane. The viscosity of the lysate was reduced by centrifugation at 13,000 rpm for 1 min. RNA binding conditions were adjusted by the addition of 350 μl of 70 % EtOH to the filtrated lysate and mixed by vortexing (2 x 5 s). The mixture was then loaded onto a NucleoSpin RNA II Column and RNA bound to the silica membrane by centrifuging the lysate at 13,000 rpm for 1 min. 350 μl Membrane Desalting Buffer were applied to the membrane and centrifuged at 13,000 rpm for 1 min, improving the efficacy prior to rDNase digestion by salt removal.

Contaminating DNA bound to the silica membrane, was removed by rDNase solution, directly applied to the membrane during the preparation and incubated at room temperature for 15 min. The sample was washed with buffer RA2 to inactivate the rDNase and subsequently washed twice with buffer RA3. Pure RNA was finally eluted under low ionic strength conditions with RNase-free H_2O.

The concentration of samples was then determined by measuring the absorbance at A_{230}, A_{260} and A_{280} using NanoDrop® ND-1000 UV-Vis Spectrophotometer, which enables highly accurate analyses of extremely small samples with remarkable reproducibility. Readings were deemed significant if $A_{260} \geq 0.1$ (1 absorbance unit at 260 nm corresponds to 40.0 µg/ml RNA). The A_{260}/A_{280} and the A_{260}/A_{230} ratios provided an estimate of the purity of the RNA, which was considered uncontaminated if both absorbance ratios were in the range of 1.9 – 2.1.

2.2.7.2 Complementary DNA (cDNA) synthesis.

cDNA was synthesized by mixing 1.0 µg of DNase-treated total RNA, PolyT and Random Primers in a total volume of 20.0 µl (Promega) using the ImpromII Reverse Transcription System. Briefly, RNA and the oligonucleotides were incubated at 70°C for 5 min to allow denaturation of RNA secondary structures and primers were hybridized to the RNA by cooling the samples to 4ºC for 5 min. Reaction buffer, $MgCl_2$, dNTPs and Reverse Transcriptase (RT) were then added and incubated at 42ºC for $\geq$ 1 h and the RT was heat inactivated at 70ºC for 15 min.

2.2.7.3 Polymerase Chain Reaction (PCR).

Polymerase chain reaction (PCR) is a method used to amplify a specific DNA sequence *in vitro* by repeated cycles of synthesis with specific primers and thermostable DNA polymerase (Saiki *et al*., 1988). Specific primers are complementary to sequences that lie on opposite strands of the template DNA and flank the segment of DNA to be amplified. Template DNA was first denatured by heating the reaction to 95ºC in the presence of a large molar excess of each of the two primers and dNTPs. The reaction mixture was cooled to allow the primers to anneal to their target sequences, and subsequently the annealed primers extended at 72ºC by Taq DNA polymerase.

Primer sequences for each gene, together with their specific T_m and the resulting fragment size are outlined in Table 2.9. Synthetic oligonucleotides were purchased from MWG Biotech AG, Ebersberg, Germany. Cycles of denaturation, annealing, and DNA synthesis were repeated 25 times as outlined below (Table 2.10). The PCR with *Taq* polymerase was used for gene screening procedures, where the expression of key genes was checked with specific primers. GoTaq®Green Master Mix (Promega) and cDNA amplification performed with annealing temperature set to be equal to that of the primer with lower T_m.

Table. 2.9 Oligonucleotides used in this study.

Name	Nucleotide Sequence 5'-3'	Length [bp]	Tm [Cº]	Fragment Size [bp]
β-actin_FWD	GTC CCG GCA TGT GCA A	16	54.3	550
β-actin_REV	AGG ATG TTC ATG AGG TAG T	19	52.4	
MC1R_FWD	GCT GGA CAA TGT CAT TGA CG	20	57.3	497
MC1R_REV	AGT TCT TGA AGA TGC AGC C	19	54.5	
MC3R_FWD	GTC TTT CCT GTG AGC AGC A	19	56.7	820
MC3R_REV	CCA GCA GAA GAT GAA CAC	18	53.7	
MC5R_FWD	GCC ATC ACG CCG GCA TC	17	58.7	340
MC5R_REV	GAG ACA TGA AGC GAG AGC	18	71.6	
GAPD_FWD_BOV	AGA ACG GGA AGC TTG TCA TC	20	57.0	743
GAPD_REV_BOV	TGA GCT TGA CAA AGT GGT CGT	21	58.0	
MC1R_FWD_BOV	CCA CCC TCC CCT TCA CCC TGG	21	67.6	301
MC1R_REV_BOV	CAT TGT CCA GCT GCT GCA CCA CG	23	66.0	
MC5R_FWD_BOV	GTC CAG AAT GCA TCC TCA CTA TGT GAG G	28	66.6	642
MC5R_REV_BOV	CAG GGT GAT GGC GCC CTT CAC G	22	68.0	
IL6_FWD	CTC AGC CCT GAG AAA GGA GA	20	59.4	450
IL6_REV	TGC AGG AAC TCC TTA AAG CTG	21	57.9	
IL8_FWD	ATG ACT TCC AAG CTG GCC GTG GCT	24	66.1	299
IL8_REV	TTA TGA ATT CTC AGC CCT CTT CAA AAA	27	58.9	
MMP1_FWD	CGA CTC TAG AAA CAG AAG AGC AAG A	25	61.3	786
MMP1_REV	AAG GTT AGC TTA CTG TCA CAC ACG CTT	27	63.4	
MMP3_FWD	GGA AAT CAG TTC TGG GCT ATA CGA GG	26	64.8	301
MMP3_REV	CCA ACT GCG AAG ATC CAC TGA AGA AG	26	64.8	

MMP9_FWD	TGT ACG GAC CCG AAG C	16	54.3	**322**
MMP9_REV	CCG TCC TTA TCG TAG TCA G	19	56.7	
MMP13_FWD	GTG GTG TGG GAA GTA TCA TCA	21	57.9	**330**
MMP13_REV	GCA TCT GGA GTA ACC GTA TTG	17	57.9	
COL1A1-FWD	ACA TGC CGA GAC TTG AGA CTC A	22	60.3	**86**
COL1A1-REV	CGA TCC ATA GTA CAT CCT GGG TTA GG	26	62.2	
COL2A1-FWD	AGC AGG TTC ACA TAT ACC GTT CTG	24	60.6	**73**
COL2A1-REV	CGA TCA TAG TCT TGC CCC ACT T	22	59.9	

Table 2.10. Master mix and program for PCR with *Taq* polymerase.

Component	Volume (µl)	Final Conc.	PCR Step	Time (min)	T (ºC)
GoTaq Green Mastermix, 2x	12.5	1x	Initial denaturation	5:00	95
Forward Primer, 10 µM	2.0	1 µM	Denaturation	1:00	95
Reverse Primer, 10 µM	2.0	1 µM	Annealing	1:30	55-59
cDNA Template	2.0	<250 ng	Elongation	1:30	72
Nuclease-Free H$_2$O	6.5	N/A	Final Elongation	10:00	72
Final Volume	**25**	**N/A**	Soak	∞	4

2.2.7.4   DNA separation by agarose electrophoresis.

Linear double-stranded DNA-fragments were separated by an electric field due to the fact that the migration of DNA varies as the reciprocal of the logarithm of the base-pairs number (Meyers *et al.*, 1976). Upon addition of ethidium bromide, which is intercalated into GC–pairs, DNA becomes fluorescent and can be visualized under UV-light. Agarose gels (2.0 %) were prepared in TBE buffer (40 mM Tris-acetate, 1.3 mM EDTA–Na, 0.47 mM CH_3COOH). Agarose gels (2 %) were prepared in TBE–buffer and DNA samples loaded onto the gel together with positive (β–actin) and negative controls and separated at 80–150 V. DNA fragments' size was determined by comparing to a DirectLoad™Step Ladder 50 bp (Sigma-Aldrich, Poole, Dorset, UK), suitable to estimate the size of DNA fragments in the range of 50–3000 bp. After electrophoresis the gel was stained in ethidium bromide (10.0 µg/ml) in TBE-buffer for 5-15 min. and visualized under UV light

2.2.7.5   Protein extraction.

There are two broad categories for complementary analytical applications of extracted proteins: identification and quantification studies and functional studies, including enzymatic assays and binding studies. Here, quantification studies have been carried out in order to determine the amount of proteins following stimulation of chondrocytes with various agents. Quantity determination is based on protein sequencing or antibody binding and does not require preservation of secondary, tertiary and quaternary structures.

Following stimulation of C-20/A4 cells with various stimuli (detailed explanation in 2.2.6.2) cell supernatants were removed and 0.5 ml of hot lysis buffer (10% v/v glycerol, 62.5 mM Tris–HCl – pH 6.8, 2% SDS, 100 mM DTT; 65º C) was added to each well. Cells were removed by scraping them off the plate using a cell scraper and lysate was collected and passed through a 25 gauge needle in order to disrupt the cell membranes. This method denatures the proteins by solubilisation with the anionic detergent Sodium Dodecyl Sulphate (SDS). Protein extracts were separated on SDS–PAGE followed by Western Blotting.

Concentrations of the protein extracts were determined at A_{280} using NanoDrop® ND-1000 UV-Vis Spectrophotometer. Briefly, the spectrophotometer was blanked with 2 μl Hot Lysis Buffer and 2.0 μl of each of the protein samples applied and the concentration and purity measured.

2.2.7.6 Sodium Dodecyl Sulphate – Polyacryl Amide Gel electrophoresis: SDS-PAGE.

SDS binds to proteins with a constant ratio of 1.4:1 (μg/μg) and thereby confers negative charges to the proteins, which increases proportionally to size and leads to linearization of the protein. Depending on the size of the molecules to be separated, electrophoresis is usually performed using either agarose (large molecules) or polyacrylamide (small molecules) gels. PAGE gels are generated by radical polymerization of acryl amide (AA) and bis–acryl amide (BA) catalysed by radicals set free from ammonium persulphate.

A discontinuous electrophoretic gel/buffer system was employed using the electrophoresis apparatus Novex® Minicell (Invitrogen, Paisley) for SDS–PAGE. Readymade NuPAGE® 10 % Bis–Tris gels of 1.0 mm x 10 wells were used. Protein samples (40 μg/lane) were mixed 1:4 in NuPAGE® Novex LDS Sample Buffer (4x; Invitrogen, Paisley; containing 4.0 % v/v β–ME), boiled for 5 mins at 100°C and loaded onto the NuPAGE® 10.0 % Bis–Tris gels. Full Range Rainbow Molecular Weight Marker (Invitrogen, Paisley) was used as a molecular weight marker. The upper buffer electrophoresis chamber was filled with 200 ml of 1x NuPAGE® Novex MOPS SDS Running buffer (20x; Invitrogen, Paisley) and the electrophoresis run at 200 V (constant), 100 mA/gel for 30 mins until the tracking dye reached the bottom of the separating gel.). The gel was washed three times with ddH$_2$O and then incubated for 30 mins in GelCode Blue Stain Reagent to visualize proteins.

2.2.7.7 Western Blot.

Following electrophoresis by SDS–PAGE, proteins were electrotransferred to 0.45 microns Hybond™–C Extra nitrocellulose membrane (Amersham Biosciences UK Ltd., Buck). The electroblotting was performed using 1 x diluted

NuPAGE® Novex Transfer buffer (20x; Invitrogen, Paisley) with added 10.0% v/v CH$_3$OH in an XCell II™ Blot Module (Invitrogen, Paisley).

Following electrotransfer, protein migration onto the membrane was checked using Ponseau S Staining Solution (0.1% w/v Ponseau S, 5.0 % v/v CH$_3$COOH). This is a rapid and reversible staining method for locating protein bands on Western blots.

The membrane was then blocked for ≥30 mins at room temperature with 10 ml of 5 % w/v skimmed milk in TBST buffer (block solution) block solution (1% v/v 1.0 M Tris–HCL pH 8.0, 3.0 % 5.0 M NaCl, 0.5% v/v Tween–20 in ddH$_2$O). Subsequently, the membrane was washed 3 x 5 mins in TBST buffer and incubated for 1.5 h at room temperature (or overnight at 4°C) with specific to the protein of interest primary antibody as detailed below (Table 2.11) in milk–TBST solution. The unbound antibody was then removed by washing the membrane 3 x 5 mins in TBST solution at room temperature. The membrane was then incubated with milk–TBST solution containing secondary antibody (polyclonal goat anti–mouse Immunoglobulins/HRP, polyclonal goat anti–rabbit Immunoglobulins/HRP) at a dilution 1:2000 v/v at room temperature for ≥ 45 mins (Table 11). Following incubation the membrane was washed in TBST for 3 x 5 mins at room temperature.

Proteins were then visualized using a chemiluminescent detection method to detect peroxide labelled secondary antibody using Pierce® ECL Western Blotting Substrate, according to manufacturer instructions. Briefly, Pierce® ECL Western Blotting Substrate (1:1 Pierce® ECL Reagent 1: Pierce® ECL Reagent 2) was directly applied to the membrane and incubated at room temperature for 5 min, after which the membrane was dried and positioned in a film–developing Hypercassette™ (Amersham Pharmacia Biotech, Buckinghamshire, UK). In a dark room, the membrane was exposed to FUJI Medical X–Ray film for 1–2 sec at room temperature. The film was subsequently developed by incubation for 3 mins in KODAK GBX developer/replenisher solution (Sigma–Aldrich, Poole), subsequent washing in ddH2O and then fixation in KODAK GBX fixer/replenisher solution (Sigma–Aldrich, Poole). Densitometric analysis was performed on the detected bands using image J analysis software.

Table 2.11 Immuno-blotting antibodies: dilutions and conditions.

Primary Antibody	Dilution	Conditions	Secondary Antibody	Dilution
α-tubulin		1h at RT	Goat anti-mouse HRP-conjugated IgG	
MC$_1$	1:2000	18 h at 4 °C		1:2000
MC$_3$		18 h at 4 °C	Goat anti-rabbit HRP-conjugated IgG	
HO-1		18 h at 4 °C		
Cleaved Caspase-3	1:2000	18 h at 4 °C	Goat anti-rabbit HRP-conjugated IgG	1:2000

In most instances, the membranes were re-probed for the detection of other proteins or secondary proteins such as the standard housekeeping protein α-tubulin. This was achieved by stripping bound antibodies on the nitrocellulose membrane with the commercially available Pierce Restore PLUS Western Blot Stripping Buffer (Thermo Fisher Scientific Inc.) at room temperature for 15 min at 37°C for high affinity antibodies. The membranes were then washed in TBST 3 x 5 min and subsequently blocked in 5 % milk blocking solution for 30 mins at room temperature prior to immuno-blotting. Sufficient removal of antibodies was ensured by incubating the stripped membranes with Pierce® ECL Western Blotting Substrate and consequently exposing them to films.

2.3 Biochemical and analytical methods.

2.3.1 Griess nitrite accumulation assay.

Griess reaction assay (Bartholomew, 1984) is a colorimetric method for determination of iNOS activity in cells following stimulation, it essentially identifies nitrite accumulation within cell culture medium. The assay is based on a chemical reaction utilising sulphanilamide and N–1–napthylethylenediamine (NED) under acidic condition (H_2PO_4), whereby nitrite interacts with sulphanilamide in a diazotization reaction forming a diazonium species. Following this the diazonium species binds to NED to form a purple azo compound that can be detected

spectrophotometrically at the dual wavelength of 560 nm and 630 nm, Thereby, provides an indication of nitrite presence in the culture medium.

Cell-free culture supernatants (100 µl) were collected from the experimental plate and transferred to a 96 well plate before an equal volume of Griess reagent (1:1 mixture of 1.0 % w/v sulphanilamide in 5.0 % aqueous H_2PO_4 and 0.1 % w/v aqueous NED) was added to each well. The plate was incubated for 10 min at room temperature for colour development. Following incubation the absorbance of the samples was measured at wavelength of A_{540} nm.

Quantification of NO_2^- levels was achieved by comparing the experimental results to a calibration curve. Only standard curves (0 – 1000 µM sodium nitrite in complete medium) with correlation coefficient $R^2 \geq 0.98$ were used to analyze experimental data, as to ascertain that the fitted model explains at least 98 % of the variation in the response variable x (Figure 2.3).
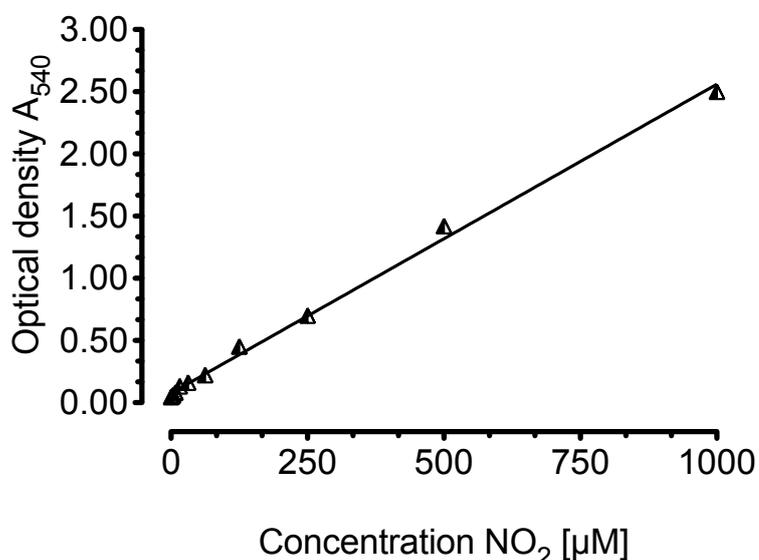
Figure 2.3 Example of a typical Griess nitrite accumulation assay standard curve.
Absorbance values for each sample were measured at 540 nm and nitrite accumulations determined from the standard curve using excel software.

2.3.2   Cytokine ELISA.

C-20/A4 and primary chondrocytes were treated at different concentrations with a panel of pro–inflammatory stimuli as describe in section 2.2.6.2. Cell-free supernatants were analysed for pro-inflammatory cytokines (IL–1β, IL–6, IL–8, IL–10 MCP–1). The method of cytokine detection was performed using commercially available ELISA kits (R&D Systems). Firstly, a 100 μl capture antibody specific to the analyte of interest was bound to a 96–well plate overnight in order to create a solid phase. Excess, unbound antibodies were washed off with Wash Buffer and incubated with 300 μl blocking reagent (1% BSA dissolved in PBS) in order to reduce any unspecific binding. The plate was further washed prior to the addition of 100 μl standards, controls and samples were incubated for 2 h to allow the analyte to bind to the capture antibody, any unbound analyte was removed by washing. A detection antibody (100 μl) was then added that binds to a different epitope and incubated for 2 h. Unbound detection antibody was removed by washing and 100 μl Streptavidin–HRP added for 20 mins. The plate was then washed and 100 μl of substrate solution TMB/H_2O_2 added and incubated for 20 mins. Colour development occurred over this period and was proportional to the amount of bound analyte. The colour development was stopped with the addition of 2N H_2SO_4 and intensity of the colour measured at A_{450} nm with wavelength correction. Quantification of cytokine levels was achieved by comparing the experimental results to a standard curve (Figure 2.4).
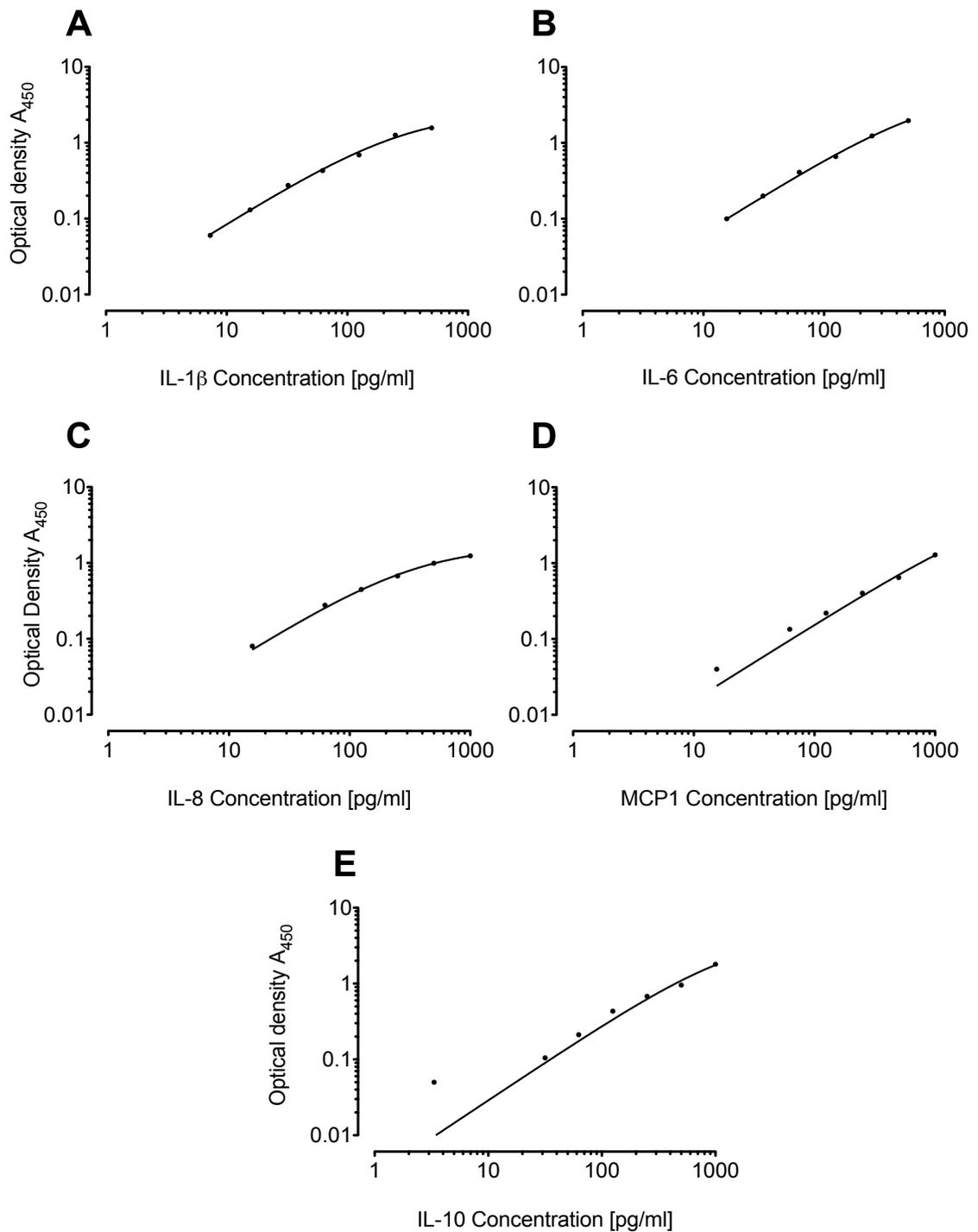
Figure 2.4 Represntative ELISA calibration curves generated using IL-1β (A), IL-6 (B), IL-8 (C), MCP-1 (D) and IL-10 (E).

The standard curves were calculated using a computer-generated 4-PL curve fit. Only standard curves with correlation coefficient R ≥ 0.98 were used to analyse experimental data, as to ascertain that the fitted model explains at least 98 % of the variation in the response variable x.

2.3.3   Cyclic AMP (cAMP) detection assay.

In functional studies, the alteration of Gi- and Gs-coupled GPCRs is normally examined by detecting the concentration of cAMP. cAMP is the intracellular signalling molecule 3'–5'–cyclic adenosine monophosphate. It modulates intracellular processes by binding to the regulatory units of different protein kinases, thus activating their catalytic subunits. That causes initiation of protein phosphorylation, an event that alters the functionality of the targeted enzymes and/or transcription factors. cAMP is formed by a chemical reaction catalysed by adenylate cyclase, which converts ATP to cAMP and inorganic phosphate and is triggered or inhibited as a result of direct interaction with G-protein α–subunits. Gs and Gi–coupled GPCR activation/inhibition with various agonist or antagonists would therefore result in increase or inhibition of cAMP production, which can be detected using the cAMP assay.

In order to determine if the melanocortin agonists are able to cause cAMP accumulation within chondrocytes, cAMP Biotrak EIA system was used. Human chondrocytic cells C-20/A4 were re–suspended to 1.0×10^5 cells/ml and plated in 96–well plates overnight to allow adherence. Medium was then replaced with 1.0 µM IBMX (90.0 µL), and cells then incubated for 30 mins with media, melanocortin peptides or Forskolin (3.0 µM). The media was removed and 100 µl lysis buffer 1B was added to each well, the plate was then placed on a plate shaker for 10 mins to allow for lysis. The lysate standards (0–3200 fmol), substrate blank (B), non–specific c binding (NSB) and samples (100 µl each) were then prepared and added to the plate. Antiserum was added to each well except the NSB and blank wells and incubated at 3 – 5°C for exactly 2 h. Then 50.0 µl cAMP –peroxidase conjugate was added to all wells except the blank and incubated at 3 – 5 ºC for 1 h. Plate was then washed with wash buffer and 150 µl of enzyme substrate was added into all wells, and the plate incubated for 30 mins at room temperature. The colorimetric reaction was then stopped by adding 100 µl Stop 1.0 M H_2SO_4 into each well and mixing. The OD was determined at A_{450} cAMP accumulation levels were then calculated by comparing the experimental results to a standard curve (Figure 2.5).
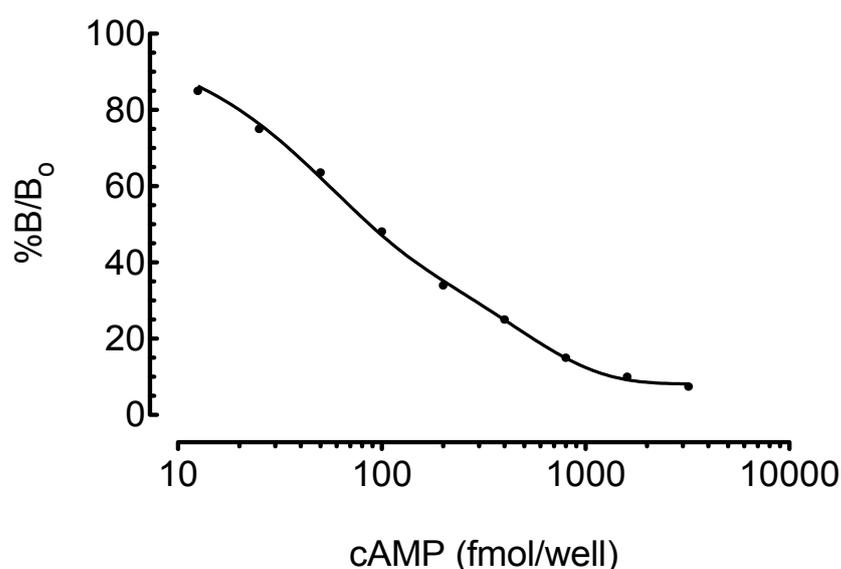
Figure 2.5 Representative Calibration Curve for cAMP accumulation.

The graph was generated by plotting the percentage B/Bo as a function of the log cAMP concentration.

2.3.4 MTT cell proliferation assay.

MTT cell proliferation assay measures mitochondrial function as an indication of cell viability (Mosmann, 1983). The assay is often used to determine the cytotoxicity of potential medicinal agents and toxic materials, since those would stimulate or inhibit cell viability and growth. The principle of this assay is to assess the ability of mitochondrial succinate dehydrogenase for metabolizing the yellow soluble form of MTT (3-(4,5-Dimethyl–2–thiazolyl)–2,5–diphenyl–2H–tetrazolium bromide, a yellow tetrazole) by reducing it to purple insoluble formazan in living cells (Figure 2.6) (Mosmann, 1983). The amount of formazan produced is directly proportional to the number of active cells and thus can be measured spectrophotometrically to quantify the formazan product.

Briefly, chondrocytes were treated with or without pro–inflammatory stimuli ± a range of melanocortin peptides/ anti–inflammatory drugs for 1 – 72h, supernatant collected and removed. Fresh media containing MTT Stock Solution (5.0 µg/ml;

10X) was added to each well and cells incubated for 2 h at 37ºC. MTT solution was then discarded and the resulting formazan deposits were dissolved in DMSO for 15 min prior to absorbance measurement at A_{570} and $A_{630.}$ Samples containing only DMEM + C-20/A4 chondrocytes represented 100 % viability (positive control), whereas 0 % was represented from DMEM without cells (negative control).
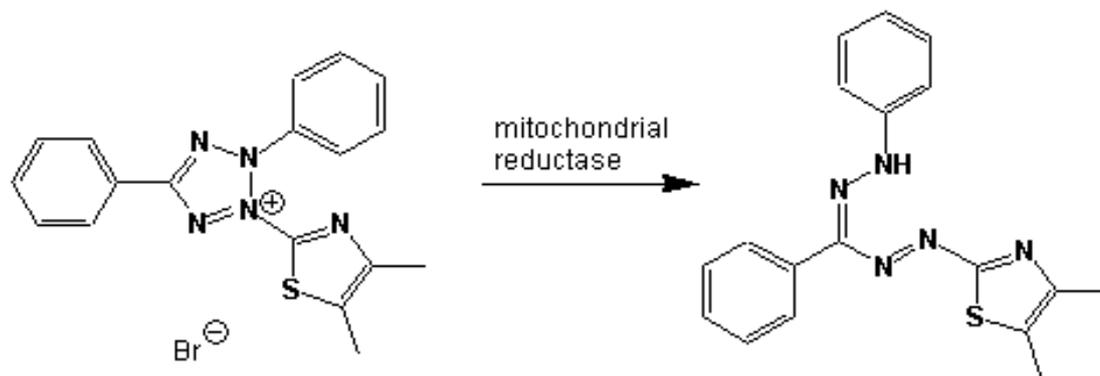
Figure 2.6 General reaction scheme for the reduction of MTT to formazan by mitochondrial succinate dehydrogenase (Mosmann, 1983).

2.3.5   Caspase-Glo 3/7 apoptosis assay.

The Caspase-Glo® 3/7 assay is a homogenous luminescent assay that measures caspase-3 and caspase-7 activities. These caspases belong to a family of cysteine/aspartic acid-specific proteases, which plays key effector roles in mammalian cells apoptosis (Nicholson and Thornberry, 1997, Thornberry and Lazebnik, 1998, Garcia-Calvo *et al*., 1999).

In order to detect cell apoptosis following various treatments of the cells, C-20/A4 cells were plated at 2.0×10^4/well in 96-well plates with 100 μl of DMEM medium supplemented with 1 % Pen/Strep and incubated for 24 h to allow adherence. Cells were treated with various agents (pro-inflammatory stimuli ± relevant drug) for 6 h and caspase 3 and 7 activity was then evaluated according to the manufacturer's protocol (Promega).

100 μl of Caspase-Glo 3/7 Reagent was added negative control cells or treated cells in DMEM medium. The blank reaction was used to measure background luminescence associated with the C20/A4 cell culture system and the Caspace-Glo reagent. The negative control reactions were prepared in order to detect and record basal levels of caspase activity in the C-20/A4 cell culture system.

Content was mixed gently on a plate shaker at 300-500 rpm for 30 sec and left at room temperature to incubate for 1 h, following which luminescence was measured using plate-reading luminometer.

2.4   Data analysis and statistics.

For both *in vitro* and *in situ* studies, experiments were performed in triplicates unless stated otherwise in the individual figure legends. Bovine articular chondrocytes were extracted and pooled together from 3 – 4 animal joints per experiment (N=3/4) and performed also in triplicate. Data are reported as mean ± standard error of the mean (SEM) unless otherwise stated. Significant differences were determined by One-way/Two-way analysis of variance (ANOVA), followed by Dunnet's post-test, or Bonferroni's multiple comparison tests as appropriate using GraphPad Prism 5.0, (GraphPad Software, CA, USA). Values of $p \leq 0.05$ were considered significant, where $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***).

Chapter 3
Results

3.1 Effect of pro-inflammatory stimuli on C-20/A4 chondrocyte function.

It is increasingly appreciated that mediators typically associated with inflammatory arthritis, such as catabolic cytokines and nitric oxide are produced by chondrocytes in OA. The role these mediators play in the progression of cartilage degradation during OA is an area of intense investigation.

In this thesis, I have evaluated whether human C-20/A4 chondrocytes might be used as a surrogate model for evaluating the effect of anti-inflammatory drugs including the effect of melanocortin peptides on inflammatory mediator release from activated chondrocytes. I have investigated the cells response to inflammatory stimuli (TNF-α, LPS, Zymosan, H_2O_2 and MSU crystals) on synthesizing and releasing pro-inflammatory mediators, such as nitric oxide and various catabolic cytokines and chemokines including IL-1β, IL-6, IL-8 and MCP-1.

3.1.1 Effect of TNF-α, LPS, H_2O_2 and MSU crystals on nitrite concentration and cell viability.

Initially, it was investigated whether a panel of stimuli (TNF-α, LPS, Zymosan, H_2O_2 and MSU crystals) were able to cause activation of the iNOS gene and this was determined by measuring the release of nitrate from stimulated chondrocytes. Nitric oxide is an important signalling molecule that acts in many tissues to regulate a diverse range of physiological processes. Cytotoxicity as a result of a substantial NO-formation is an established apoptosis initiating factor (Brune *et al.*, 1999). Following stimulation, chondrocyte viability was assessed by MTT assay to ascertain the effect of the inflammatory stimuli on this parameter.

C-20/A4 chondrocytes were plated in monolayer in DMEM media (50.0 U/ml Penicillin, 50.0 µg/ml Streptomycin) for 24 h prior to stimulation with TNF-α (20.0 – 80.0 pg/ml), LPS (0.1 – 10.0 µg/ml), H_2O_2 (0.01 – 1.0 %) or MSU crystals (0.1 – 1.0 µg/ml) for 6 h. Cell free supernatants were collected and analysed for nitrite release by Griess assay. In unstimulated cells there was a basal release of nitrite (1.227 ± 1.214 µM) and the effect of the inflammogens were compared to this

value. In initial experiments C-20/A4 chondrocytes were stimulated with high concentrations of H_2O_2 to determine whether C-20/A4 chondrocytes produce nitrite compared to unstimulated control cultures.

H_2O_2 caused a concentration-dependent increase in NO release, where 0.01%, 0.1% and 1.0% H_2O_2 led to 139.28 ± 6.24 μM, 175.67 ± 7.18 μM and 210.12 ± 10.31 μM respectively ($p \leq 0.001$), thereby indicating that the chondrocytes can release substantial quantities of nitrite when stimulated (Figure 3.1 A). After validating the ability of C-20/A4 cells to respond to oxidative stress by activating iNOS, we tested the response of the chondrocytes to TNF-α, LPS and MSU crystals stimulation.

Figure 3.1 B demonstrates the effect of TNF-α (20.0 – 80.0 pg/ml) on nitrite formation. Stimulation of the human cell-line chondrocytes led to a significant release of nitrite, whereby TNF-α (20.0 pg/ml) caused 19.18 ± 6.79 μM, which was not substantially altered upon increasing the concentrations, however, maximal release was observed at 80.0 pg/ml (20.86 ± 6.33 μM, $p \leq 0.05$ vs. control). Next, it was evaluated whether the observed effect was due to the particular stimulus being used; therefore nitrite release in the presence of LPS (0.1 – 10.0 $\mu g/ml$) was examined (Figure 3.1) and significant alterations were detected: 36.35 ± 7.95 μM, 30.35 ± 9.64 mM and 24.99 ± 7.21 μM for 1.0, 3.0 and 10.0 $\mu g/ml$ LPS respectively ($p \leq 0.05$ for all concentrations tested). Conversely, MSU crystals (30 – 1000 ng/ml) did not cause a significant increase in nitrite release compared to unstimulated controls ($p > 0.05$; Figure 3.1 D).

Following identification of C-20/A4 chondrocytes capability to produce NO, cell viability was assessed by MTT assay in the presence of these stimuli, thereby confirming the cytotoxicity of the given stimulus in this cell-line model. To ensure that cell death could be measured in C-20/A4 chondrocytes, cells were stimulated with 0.01 %, 0.1 % and 1.0 % H_2O_2, which caused a concentration-dependent decrease in cell viability with 82.25 ± 4.66 %, 87.14 ± 4.23 % and 92.80 ± 1.29 % cell death respectively ($p < 0.001$; Figure 3.2A). Similarly, TNF-α (20.0 – 80.0 pg/ml) caused a significant concentration-dependent decline in C-20/A4 chondrocyte viability (Figure 3.2 B), where 20.0 pg/ml caused 16.14 % ±

4.09 % reduction in viability ($p \leq$ 0.05). Analogous effects were noted following 40.0 pg/ml (11.8 ± 3.4%, n.s.), whilst at higher concentrations (60.0 pg/ml) there was 27.58 ± 2.85% ($p \leq$ 0.01) rate of cell death, which was slightly increased at 80.0 pg/ml to 30.22 ± 1.18 % ($p \leq$ 0.001) compared to untreated cells (Figure 3.2 B). C-20/A4 chondrocytes were then stimulated with LPS, which caused a 23.29 ± 3.14 % ($p \leq$ 0.001), 19.24 ± 2.83 % ($p \leq$ 0.001) and 17.42 ± 3.23 % ($p \leq$ 0.01) cell death at 1.0, 3.0, and 10.0 μg/ml concentrations respectively.

MSU crystals (30 – 1000 ng/ml) did not alter cell viability of C-20/A4 chondrocytes compared to unstimulated cells. This finding, combined with the observation that it did not induce nitrite release led to discontinuation of further use of this inflammogen (Figure 3.1 D, Figure 3.2 D).
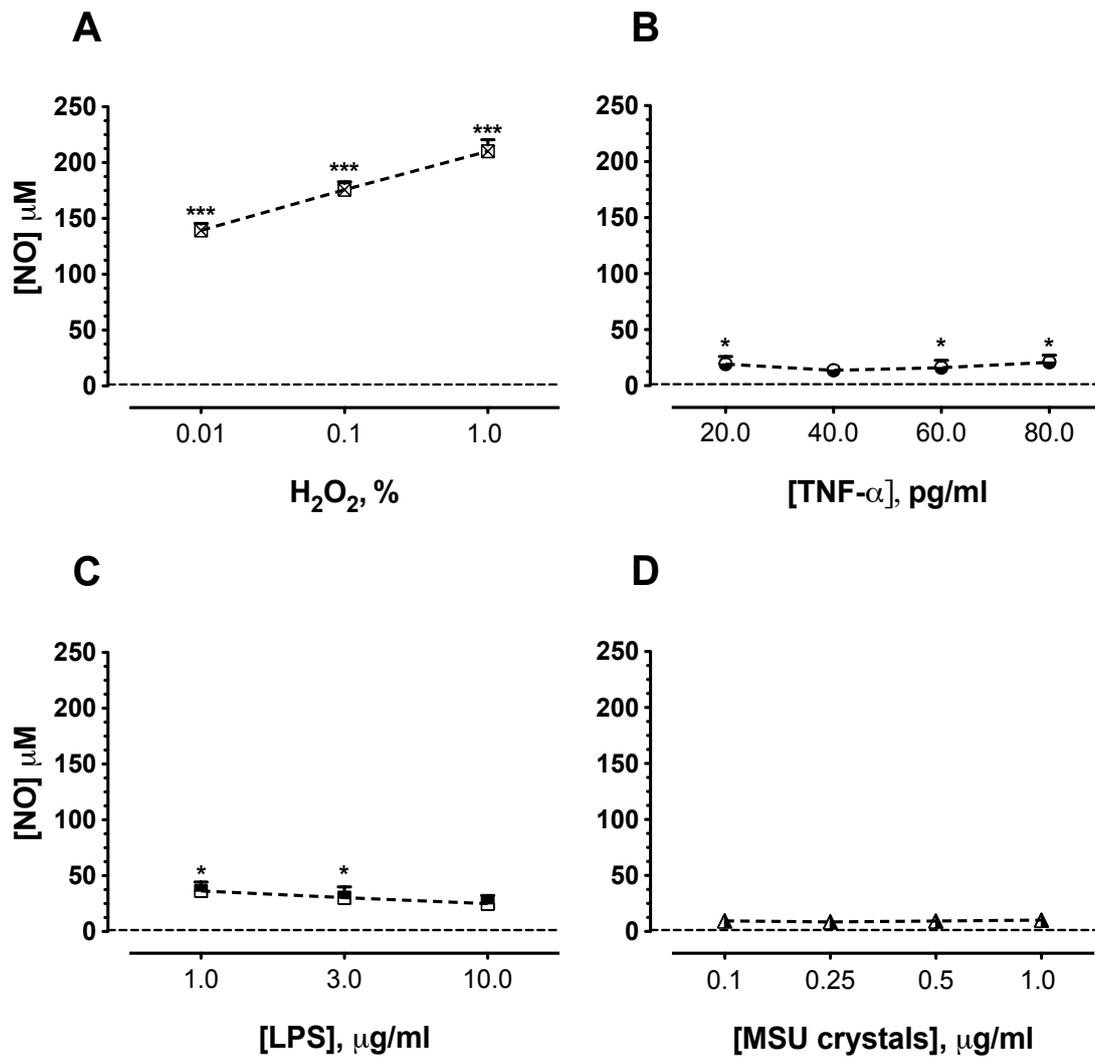
Figure 3.1. The effect of inflammatory stimuli on nitric oxide production from C-20/A4 chondrocytes.

C-20/A4 chondrocytes (1.5×10^5 cells/cm^2) were plated in serum-free DMEM and stimulated with H_2O_2 (0.01 – 1.0 %; Panel A), TNF-α (20.0 – 80.0 pg/ml; Panel B), LPS (1.0 – 10.0 μg/ml; Panel C) or MSU crystals (0.1 – 1.0 μg/ml; Panel D) for 6 h. Cell-free supernatants were then collected and analysed for nitrite release via Griess assay. Dotted line represents basal release of nitrite (1.23 ± 1.21 μM). Data are presented as Mean $\pm$ SEM of n=9 experiments, assessed in triplicate. *$p \le 0.05$, **$p \le 0.01$, ***$p \le 0.001$ vs. untreated control cultures.
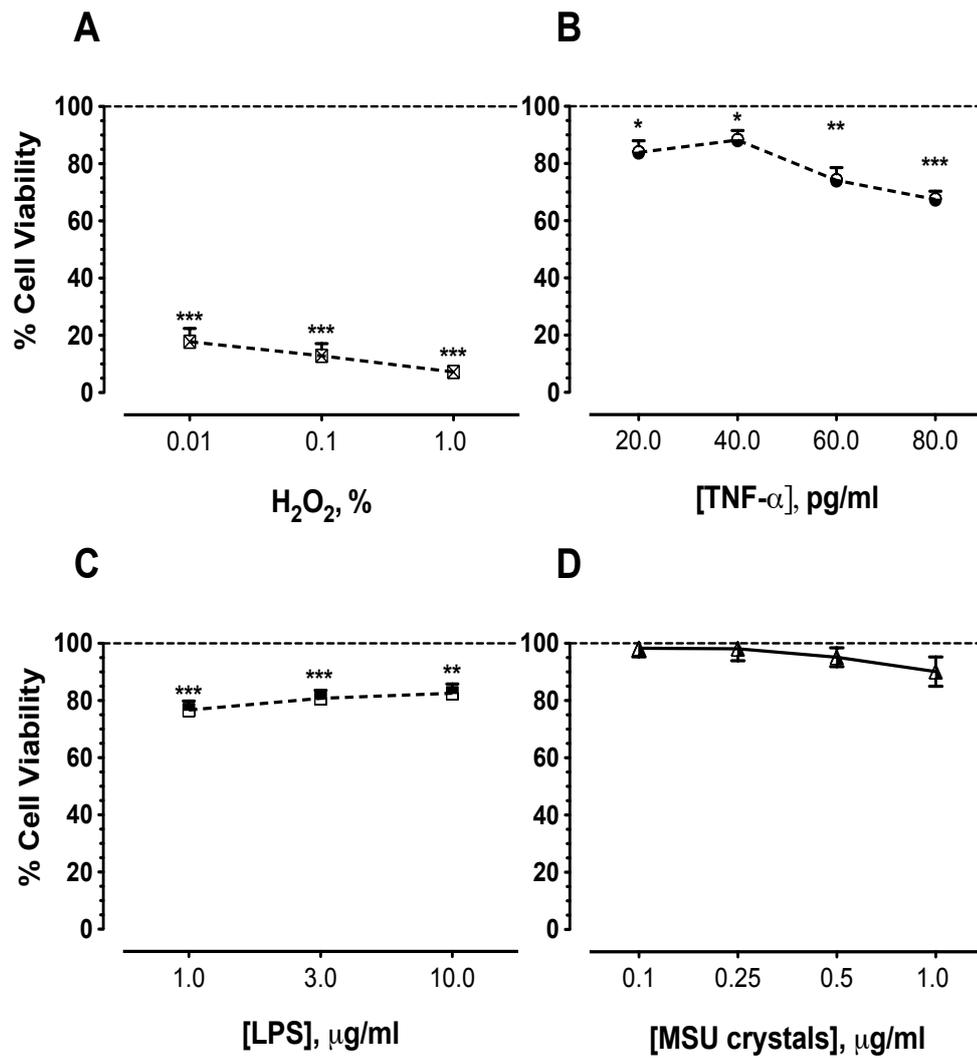
Figure 3.2. The effect of inflammatory stimuli on C-20/A4 chondrocytes viability.
C-20/A4 chondrocytes (1.5 x 10⁵ cells/cm²) were plated in serum-free DMEM (280 and stimulated with H_2O_2 (0.01 – 1.0 %; Panel A), TNF-α (20.0 – 80.0 pg/ml; Panel B), LPS (1.0 – 10.0 μg/ml; Panel C) or MSU crystals (0.1 – 1.0 μg/ml; Panel D) for 6 h. Cell viability was then determined via MTT reduction assay. Dotted line represents control sample cell viability – untreated cells (100%). Data are presented as Mean ± SEM of n=9 experiments, assessed in triplicate. *p≤ 0.05, **p≤ 0.01, ***p≤ 0.001 vs. untreated control cultures.

3.1.2 Detection of pro-inflammatory cytokines release following stimulation of C-20/A4 chondrocytes with TNF-α and LPS.

The importance of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 IL-8 in the induction of catabolic processes in chondrocytes has been recognized (Pelletier *et al.*, 1991, Shinmei *et al.*, 1991, Lotz *et al.*, 1992, Reboul *et al.*, 1996, Melchiorri *et al.*, 1998, Shlopov *et al.*, 2000, Fernandes *et al.*, 2002, Schuerwegh *et al.*, 2003, Rai *et al.*, 2008, Goldring *et al.*, 2008). The major source of pro-inflammatory cytokines in OA has been thought to be activated synovium or infiltrating inflammatory cells, however strong evidence exists that chondrocytes can also release these mediators and drive the inflammatory response within the joint (Goldring, 2000; Goldring *et al.,* 2011). In order to investigate whether human C-20/A4 chondrocytes are capable of releasing pro-inflammatory cytokines, cells were stimulated with TNF-α and LPS and mRNA and protein levels of pro-inflammatory cytokines were determined.

3.1.2.1 Effect of TNF-α and LPS on *IL6* and *IL8* mRNA expression in C20/A4 chondrocytes.

In initial experiments, the effect of TNF-α (60.0 pg/ml) and LPS (1.0 μg/ml) was investigated on *IL6* and *IL8* mRNA expression levels following 0 – 48 h incubation periods. Total RNA was extracted from stimulated and non-stimulated cells and conventional RT-PCR amplification reactions with specific primers for *IL6* and *IL8* genes (Table 2.9) were used. Gene expression was visualized on 2 % agarose gels, run in conjunction with *β-actin* (Figure 3.5 A). RT-PCR showed that C-20/A4 chondrocyte respond to TNF-α (60.0 pg/ml), a concentration chosen from previous experiments to cause a sub-maximal rate of cell death and nitrite release, thereby substantially increasing *IL6* and *IL8* expression in time-dependent manner (Figure 3.3 A). Maximal mRNA levels of *IL6* and *IL8* were detected 2 h post-stimulation gradually decreased in the period between 6 – 48 h, as determined by densitometric analysis (Figure 3.3 B and C). Incubation period of 6 h was chosen as suitable for subsequent experiments as it led to sub-maximal release of *IL6* and *IL8*, thereby allowing for the concentration dependent effect of TNF-α to be investigated. TNF-α (20.0 – 80.0 pg/ml) caused increases

in *IL6* and *IL8* with densitometric quantification showing a 53-fold increase in expression of *IL6* peaking at 40.0 pg/ml TNF-α (Figure 3.4 A), and a 9-fold increase in *IL8* compared to control (Figure 3.4 B and C).

In order to clarify the response of C-20/A4 chondrocytes to LPS, the effect of various concentrations on pro-inflammatory cytokine expression was investigated. LPS induced a concentration-dependent increase in the expression of *IL6* and *IL8* (Figure 3.4 C and D), whereby chondrocytes were notably activated following concentrations as low as 0.1 μg/ml, and reaching a 72-fold increase in *IL6* and 13.5-fold increase in *IL8* expression at 1.0 μg/ml compared to untreated controls ($p \leq 0.001$). Each value was normalized to the respective *β-actin* expression. Shown are the means of four independent experiments.
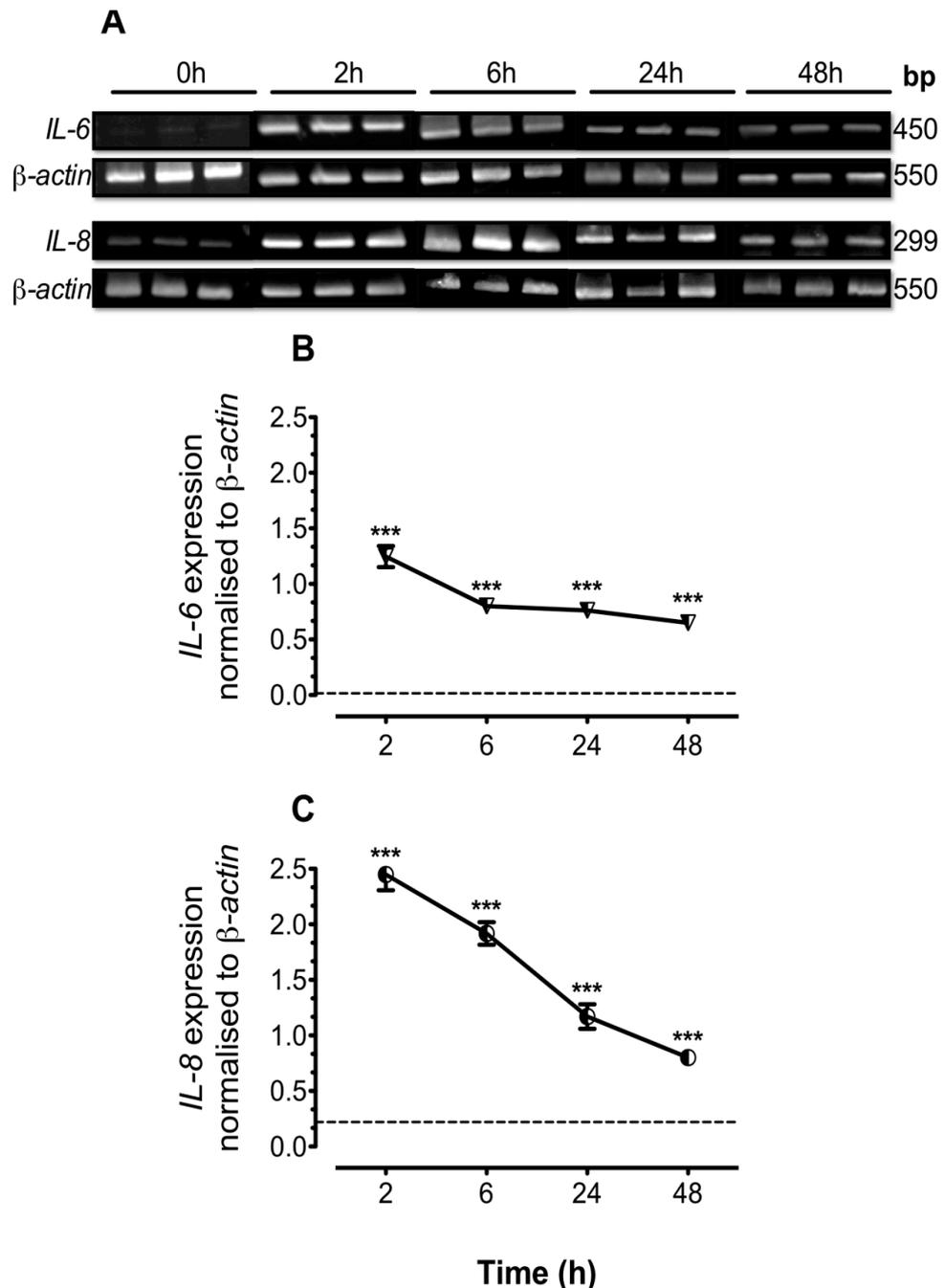
Figure 3.3. Densitometric quantification of *IL6* and *IL8* expression in C-20/A4 chondrocytes treated with TNF-α (60.0 pg/ml) for 0-48 h.

C-20/A4 chondrocytes were stimulated with TNF-α (60.0 pg/ml) at time 0 and total RNA extracted 0-48 h post-stimulation. PCR amplification with the respective primers for *IL6* and *IL8* was used to detect and quantify gene expression on 2% agarose gels in triplicates with β-actin used as internal control (Panel A). Comparison of densitometrically quantified *IL6* (Panel B) and *IL8* (Panel C) shown in arbitrary units, each value normalized to the respective *β-actin* expression. Data are presented as Mean ± SEM of four independent experiments *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

Figure 3.4. Densitometric quantification of *IL6* and *IL8* expression in C-20/A4 cells treated with TNF-α (20.0 – 80.0 pg/ml) and LPS (0.1 – 1.0 µg/ml) for 6 h.

C-20/A4 chondrocytes were stimulated with TNF-α (20.0 – 80.0 pg/ml) and LPS (0.1 – 1.0 µg/ml) at time 0 and total RNA extracted 6 h post stimulation. PCR amplification with the respective primers for *IL6* and *IL8* was used to detect and quantify gene expression on 2 % agarose gels in triplicates with *β-actin* used as internal control (Panel A). Comparison of densitometrically quantified *IL6* (Panel B and C) and *IL8* (Panel D and E) shown in arbitrary units, each value normalized to the respective *β-actin* expression. Data are presented as Mean ± SEM of four independent experiments *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

3.1.2.2 Effect of TNF-α and LPS on IL-6 and IL-8 cytokine release from C20/A4 chondrocytes.

Following identification of TNF-α and LPS ability to induce *IL6* and *IL8* expression in C-20/A4 chondrocytes, the effect of various concentrations of these stimuli over a time course was investigated, thereby confirming the genuine translation of *IL6* and *IL8* mRNA to protein. C-20/A4 chondrocytes were stimulated with TNF-α (20.0 – 80.0 pg/ml) and LPS (1.0 – 10.0 μg/ml) for 0, 2, 6 and 24 h and cell-free supernatants were collected and analysed. Stimulation for 2 h with TNF-α (20.0 – 80.0 pg/ml) resulted in well-defined bell-shaped curve effect in IL-6 release, peaking at 60.0 pg/ml (99.23 ± 13.81 pg/ml; $p \leq 0.05$) and decreasing thereafter with 80.0 pg/ml leading to 74.55 ± 17.89 pg/ml ($p \leq 0.05$; Figure 3.5 A). Lower concentrations of TNF-α failed to illicit significant IL-6 release at this time-point as compared to control (DMEM treated cultures). Subsequently, a longer, 6 h incubation period was employed to investigate IL-6 production from chondrocytes in response to TNF-α, with 20.0 pg/ml and 40.0 pg/ml of TNF-α caused 45.52 ± 8.3 pg/ml and 59.64 ± 2.4 pg/ml of IL-6 respectively ($p \leq 0.05$; Figure 3.5 B). As previously seen, maximal release following 2 h of stimulation was caused by 60.0 pg/ml of TNF-α with 117.1 ± 26.09 pg/ml ($p \leq 0.05$), which slightly decreased following treatment with 80.0 pg/ml TNF-α (84.31 ± 5.69 pg/ml; $p \leq 0.05$). Analogous effects were observed at 24 h, when a bell-shaped release of IL-6 was detected with 20.0 pg/ml and 40.0 pg/ml causing 52.79 ± 2.60 pg/ml and 63.30 ± 6.97 pg/ml of IL-6 respectively ($p \leq 0.05$). Increasing the concentrations led to the detection of a maximal release of 154.30 ± 10.32 pg/ml ($p \leq 0.01$) detected at 60.0 pg/ml, which decreased at 80.0 pg/ml TNF-α (139.34 ± 11.48 pg/ml ($p \leq 0.01$; Figure 3.5 C).

The effect of LPS (0.1 – 10.0 μg/ml) was subsequently determined and confirmed to cause a concentration-dependent increase in IL-6 at 2 h compared to untreated control cultures (Figure 3.5 D). Concentrations lower than 1.0 μg/ml did not cause statistically significant difference compared to control values ($p \leq 0.05$), but higher concentrations of LPS led to 73.467± 13.83 pg/ml, 176.85 ± 23.5 pg/ml and 152.34 ± 6.44 pg/ml of IL-6 production ($p \leq 0.05$) for 1.0, 3.0 and 10.0 μg/ml respectively. Similar observations were made when C-20/A4 chondrocytes were

treated with LPS for 6 h; however, here, in accordance with RT-PCR detected levels, 0.1 μg/ml and 0.3 μg/ml of LPS caused a significant increase in IL-6 production, leading to 61.33 ± 7.11 pg/ml and 68.67 ± 10.33 pg/ml respectively ($p \leq$ 0.05; Figure 3.5 E). At 24 h post-stimulation LPS (0.1 – 10.0 μg/ml), a maximal release of 134.93 ± 14.72 pg/ml of IL-6 was recorded following stimulation with LPS (3.0 μg/ml; $p \leq$ 0.01; Figure 3.5 F) There was no significant difference between the potency of the various concentrations probably due to a plateau, which is reached at concentrations this high.

Consequently the translation of IL-8 mRNA to protein was determined. C-20/A4 chondrocytes were stimulated with TNF-α and LPS for 0, 2, 6 and 24 h and a bell-shaped response was recorded regardless of the time-point. The results, reported in Figure 3.6 A, demonstrated the lack of efficacy of low concentrations of TNF-α (20.0 and 40.0 pg/ml) at short incubation periods (2 h) in the chondrocyte system. However, 2 h-stimulation with 60.0 pg/ml and 80.0 pg/ml of TNF-α caused significant IL-8 production with 130.23 ± 9.5 pg/ml and 142.65 ± 12.5 pg/ml respectively ($p \leq$ 0.05), (Figure 3.6 A). These amounts were compared to basal levels of IL-8 synthesis (63.34 ± 5.32 pg/ml). TNF-α caused a bell-shaped response in IL-8 production at 6 h post-simulation, peaking at 60.0 pg/ml (205.90 ± 27.1 pg/ml; $p \leq$ 0.01), and slowly decreasing following stimulation with 80.0 pg/ml of TNF-α (145.68 ± 4.72 pg/ml; $p \leq$ 0.01; Figure 3.6B). In contrast to the effect at 20.0 and 40.0 pg/ml of TNF-α at 2h, these concentrations led to significant increase in IL-8 production following 6 h incubation (120.38 ± 3.67 pg/ml and 131.8.41 pg/ml; $p \leq$ 0.05; Figure 3.6 B) compared to control (72.65 ± 7.34 pg/ml). Lastly, TNF-α effect at 24 h was determined with 20.0 and 40.0 pg/m leading to 348.57 ± 53.23 pg/ml and 391.36 ± 35.7 pg/ml of IL-8 respectively ($p \leq$ 0.01), compared to control (79.33 ± 15.65 pg/ml).

LPS (0.1 – 10.0 μg/ml), being a bacterial lipopolysaccharide has been shown to cause joint and cartilage inflammation (Lotz *et al*., 1992). At all concentrations and time-points tested it caused a significant increase in IL-8 production. At 2 h, 6 h and 24 h there was a concentration-dependent increase leading to a maximal release of IL-8 at 3.0 μg/ml with 189.5 ± 25.46 pg/ml, ($p \leq$ 0.05), 243.53 ± 18.31 pg/ml ($p \leq$ 0.01) and 272.54 ± 10.29 pg/ml ($p \leq$ 0.01) respectively. All other

concentrations caused similarly significant amounts of IL-8, but were not different from one another within the incubation period (Figure 3.6 D-F).
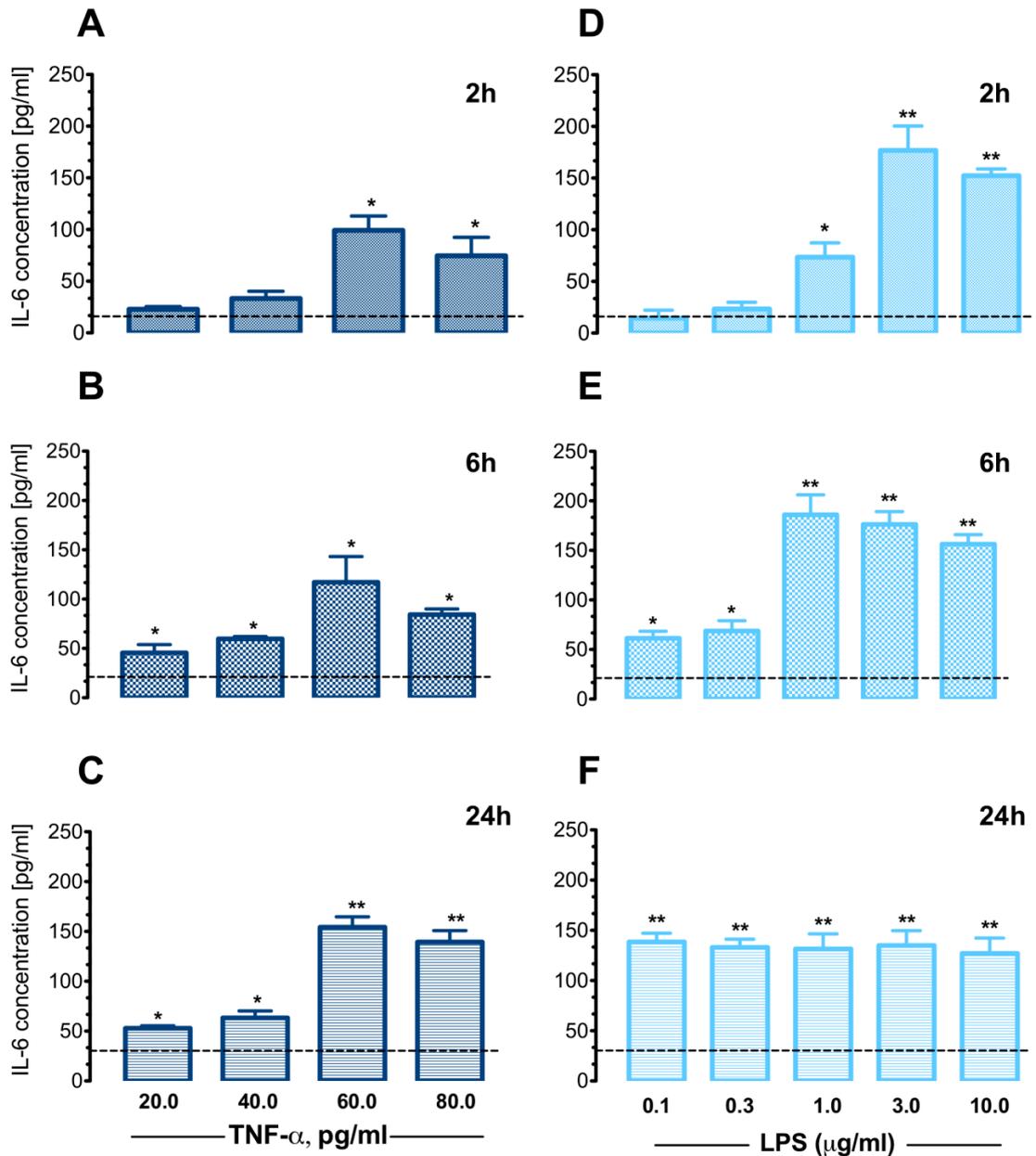
Figure 3.5 Effect of pro-inflammatory stimuli on IL-6 release from C20/A4 chondrocytes.

C-20/A4 chondrocytes were stimulated with TNF-α (20.0 – 80.0 pg/ml; Panel A – C) or LPS (0.1 – 10.0 μg/ml; Panel D – F) and cell-free supernatants collected at 0, 2, 6 and 24 h. IL-6 release was then determined by ELISA. Data are presented as Mean ± SEM of n=4 independent experiments repeated in triplicate, *p≤ 0.05, **p≤ 0.01 vs. Time 0 (dotted line).
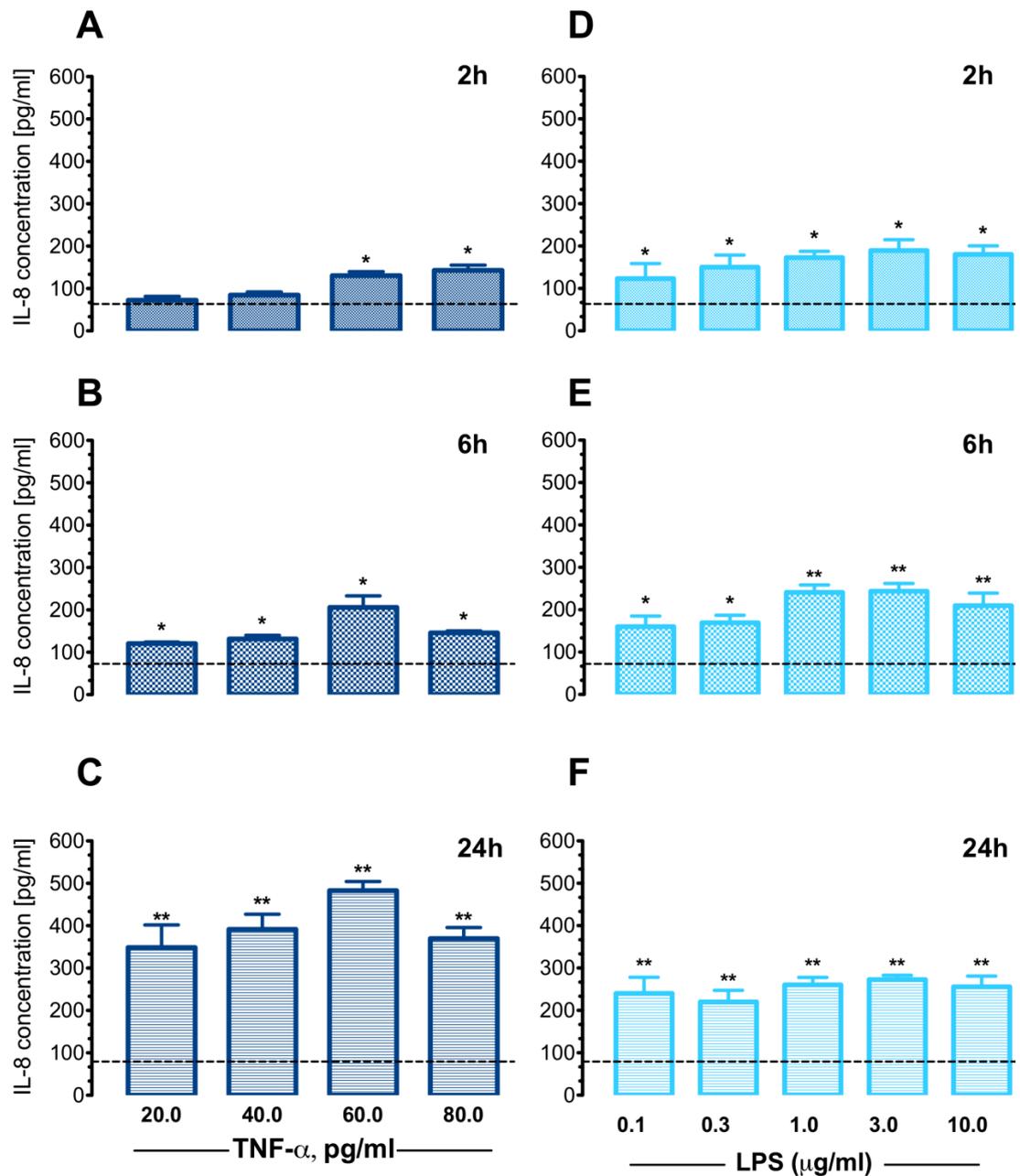
Figure 3.6. Effect of pro-inflammatory stimuli on IL-8 release from C20/A4 chondrocytes.

C-20/A4 chondrocytes were stimulated with TNF-α (20.0 – 80.0 pg/ml; Panel A – C) or LPS (0.1 – 10.0 μg/ml; Panel D – F) and cell-free supernatants collected at 0, 2, 6 and 24 h. IL-8 production was then determined by ELISA. Data are presented as Mean ± SEM of n=4 independent experiments repeated in triplicate, *p≤0.05, **p≤0.01 vs. Time 0 (dotted line).

3.1.2.3 Effect of TNF-α and LPS on IL-1β and MCP-1 cytokine release from C-20/A4 chondrocytes.

In separate experiments C-20/A4 chondrocytes were stimulated with different concentrations of TNF-α (20.0 – 80.0 pg/ml) and LPS (1.0 – 10.0 μg/ml) for 0, 2, 6 and 24 h and cell-free supernatants were analysed for IL-1β and MCP-1 by ELISA. Given that IL-1β is considered to be active locally within cartilage and leads to matrix destruction, IL-1β levels were measured (Attur *et al.*, 2000; Fernandes *et al.*, 2002; Kapoor *et al.*, 2011). In addition, to assess chondrocyte contribution to the chemotactic environment of inflamed joints the intracellular content of the CC (IL-1β) and CXC chemokine (MCP-1) was investigated, with specific interest in MCP-1 production (Villager *et al.*, 1992; Borzi *et al.*, 1999). TNF-α and LPS caused a significant increase in IL-1β release ($p \leq 0.05$) compared to unstimulated control cells. Treatment with TNF-α caused a significant concentration-dependent increase in IL-1β at 2, 6 and 24 h post-stimulation.

Following 2 h stimulation, the lower concentrations (20.0 and 40.0 pg/ml) of TNF-α didn't cause significant up-regulation in IL-1β production, consistent with the results obtained for IL-6 and IL-8. The maximal release of 16.74 pg/ml at this time point was induced by 80.0 pg/ml ($p \leq 0.05$; Figure 3.7 A), and these observations were confirmed at 6 h, where 80.0 pg/ml caused 28.35 ± 3.25 pg/ml IL-1β to be released ($p \leq 0.05$; Figure 3.7 B), However, at 6 h, 40.0 pg/ml and 60.0 pg/ml of TNF-α also led to significant increase in the concentration of IL-1β, 15.73 ± 1.1 pg/ml and 24.37 ± 8.69 pg/ml respectively ($p \leq 0.05$). IL-1β was not detectable at 24 h following stimulation with 80.0 pg/ml, however at 60.0 pg/ml, a small but detectable amount of IL-1β was observed (14.61 ± 3.1 pg/ml, $p \leq 0.05$; Figure 3.7 C).

LPS (3.0 μg/ml) stimulation of C-20/A4 chondrocytes for 2 h caused the maximal release of IL-1β for this time point with 17.23 ± 2.86 pg/ml ($p \leq 0.05$; Figure 3.7 D). Following 6 h stimulation, LPS caused a concentration-dependent bell-shaped response, with 1.0 µg/ml causing 26.55 ± 10.31 pg/ml of IL-1β to be released ($p \leq 0.05$; Figure 3.7 E). At 24 h post-stimulation, a plateau was reached,

with all concentrations causing similar amounts of IL-1β to be released (p≤ 0.05; Figure 3.7 F).

Next, TNF-α (20.0 – 80.0 pg/ml) effect was evaluated on MCP-1 production, where stimulation for 2 h (Figure 3.8 A) did not yield statistically significant increases in MCP-1 compared to basal levels (46.18 ± 14.21 pg/ml; $p≤ 0.05$). TNF-α (20.0 pg/ml) did not cause any significant change in MCP-1 levels regardless of the incubation periods ($p> 0.05$) evaluated, however, with increasing concentrations of the cytokine at 6 h (Figure 3.8 B), there was a significant increase in MCP-1 production of 69.39 ± 4.21 pg/ml ($p≤ 0.05$), 101.2 ± 16.37 pg/ml ($p≤ 0.05$) and 119.92 ± 8.74 pg/ml ($p≤ 0.05$) for TNF-α 40.0 pg/ml, 60.0 pg/ml and 80.0 pg/ml respectively. MCP-1 levels at 24 h following stimulation with TNF-α (40.0 – 80.0 pg/ml; Figure 3.8 C) followed a bell-shaped curve, where 40.0, 60.0 and 80.0 pg/ml TNF-α causing 70.43 ± 5.23 pg/ml ($p≤ 0.05$), 101.34 ± 12.09 ($p≤ 0.05$) and 93.324 ± 6.42 pg/ml ($p≤ 0.05$) respectively compared to basal production (39.29 ± 11.28 pg/ml).

Subsequently, LPS was evaluated and found to stimulate significant rise in MCP-1 release with a maximal response achieved at 3.0 and 10.0 μg/ml following 6 h incubation (Figure 3.8 E), with 68.34 ± 4.52 pg/ml and 70.11 ± 3.32 pg/ml respectively compared to untreated controls (46.18 ± 14.21 pg/ml). At 2 h post-stimulation (Figure 3.8 D), MCP-1 release was lower 25.29 ± 2.38 pg/ml (0.3 μg/ml; $p≤ 0.05$), 41.34 ± 12.23 pg/ml (1.0 μg/ml; $p≤ 0.05$), 23.55 ± 6.89 pg/ml (3.0 μg/ml, $p≤ 0.05$) and 32.43 ± 12.48 pg/ml (10.0 μg/ml; $p≤ 0.05$). Incubation periods longer than 6 h did not induce statistically significant increases in MCP-1 release from C-20/A4 chondrocytes compared to basal levels (39.31 ± 8.89; $p≤ 0.05$; Figure 3.8 F).
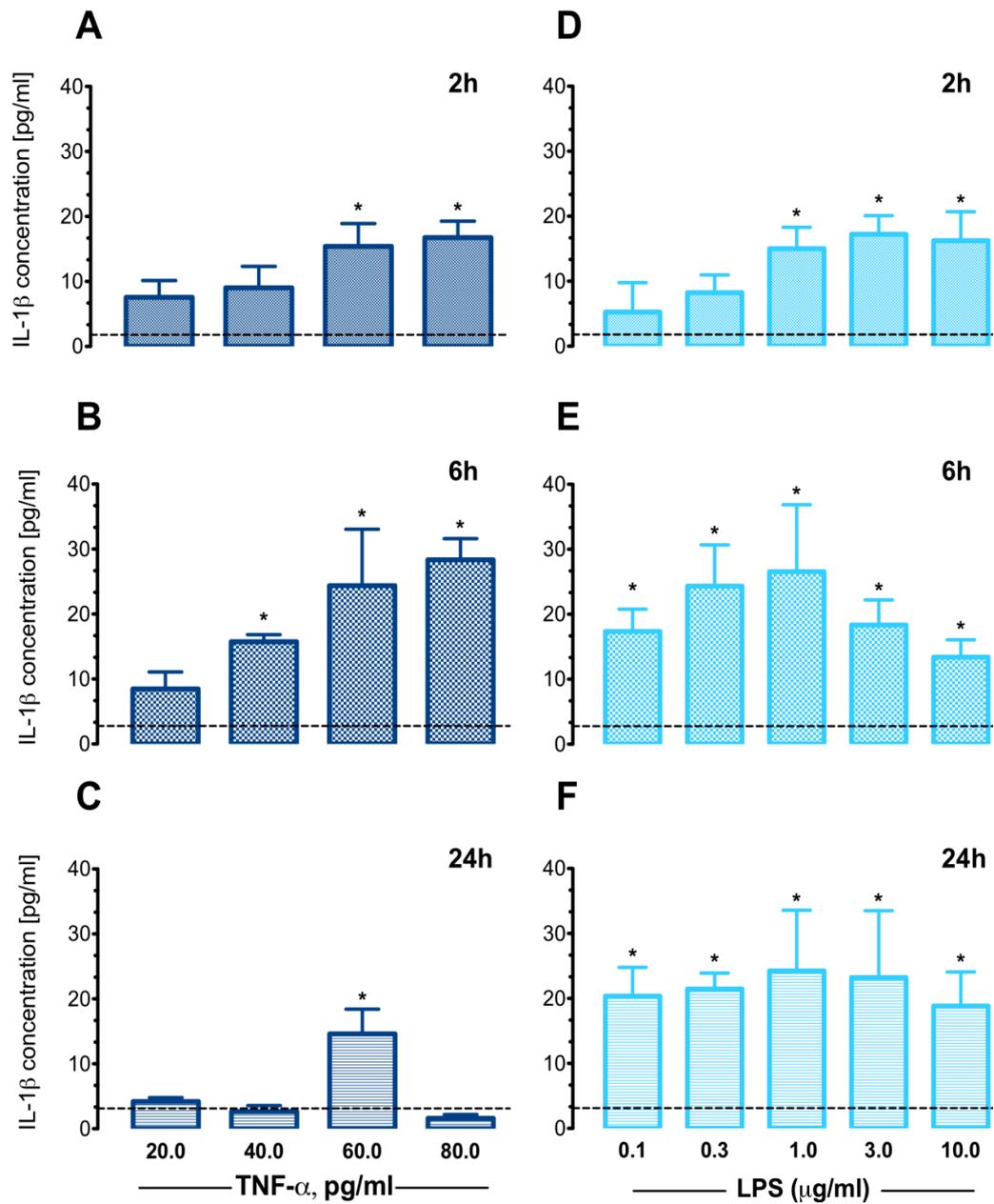
Figure 3.7. Effect of pro-inflammatory stimuli on IL-1β release from C20/A4 chondrocytes.

C-20/A4 chondrocytes were stimulated with TNF-α (20.0 – 80.0 pg/ml; Panels A – C) or LPS (0.1 – 10.0 μg/ml; Panels D – F) and cell-free supernatants collected at 0, 2, 6 and 24 h and IL-1β determined by ELISA. Data are presented as Mean ± SEM of n=4 independent experiments repeated in triplicate, *p≤ 0.05 vs. Time 0 (Dotted line).
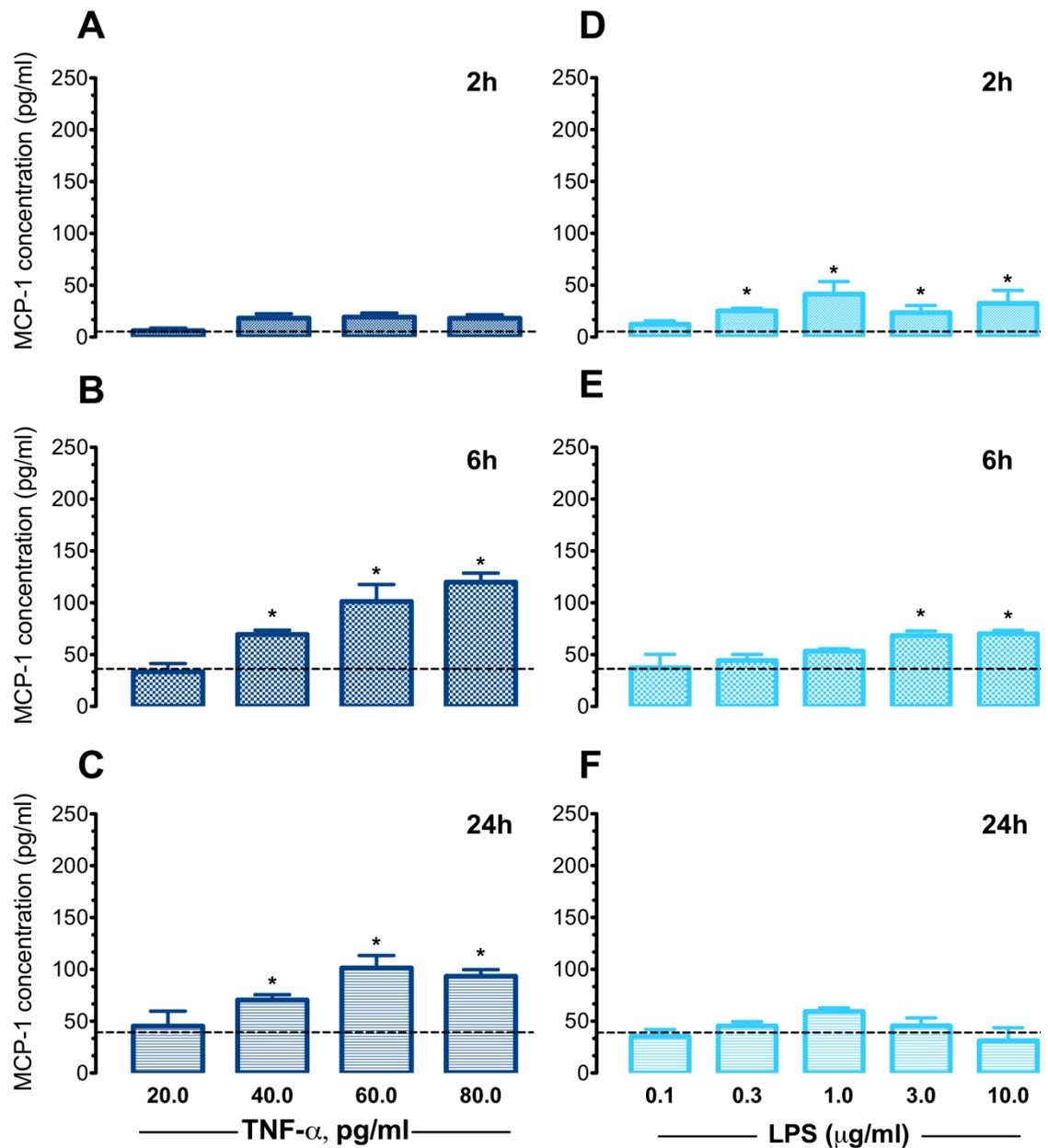
Figure 3.8. Effect of pro-inflammatory stimuli on MCP-1 release from C20/A4 chondrocytes.

C-20/A4 chondrocytes were stimulated with TNF-α (20.0 – 80.0 pg/ml; Panel A – C) or LPS (0.1 – 10.0 μg/ml; Panel D – F) and cell-free supernatants collected at 0, 2, 6 and 24 h and analysed for MCP-1 by ELISA. Data are presented as Mean ± SEM of n=4 independent experiments repeated in triplicate, *$p \leq$ 0.05 vs. Time 0 (Dotted line).

3.1.2.4 Time dependent release of pro-inflammatory cytokines following C-20/A4 chondrocyte stimulation.

In order to fully understand the response of C-20/A4 chondrocytes to inflammogens, they were stimulated with TNF-α at 60.0 pg/ml and LPS at 1.0 µg/ml (the most potent concentrations identified from previous experiments) for a longer time course investigation including incubation periods of up to 72 h. Cell-free supernatants were collected at each individual time point and subsequently analysed for IL-6 and IL-8 release by ELISA.

Cultured C-20/A4 cells had a basal release of IL-6 (21.09 ± 18.49 pg/ml) and IL-8 (72.65 ± 13.32 pg/ml). TNF-α (60.0 pg/ml) and LPS (1.0 µg/ml) caused a significant time-dependent increase in IL-6 (Figure 3.9 A, B) and IL-8 (Figure 3.9 C, D), with maximal production detected at 48 h post-incubation, with 154.30 ± 10.32 pg/ml and 159.65 ± 20.86 pg/ml ($p \le 0.001$) of IL-6 following TNF-α and LPS stimulation respectively. Maximal release of IL-8 was detected at 48 h (558.90 ± 11.34 pg/ml, $p \le 0.001$), whilst following LPS stimulation it was detected at 72 h (378.79 ± 46.86 pg/ml, $p \le 0.001$). Release of these pro-inflammatory cytokines following 2 h of incubation with TNF-α (60.0 pg/ml) and LPS (1.0 µg/ml) caused significant increases of 99.23 ± 13.81 pg/ml ($p \le 0.05$) and 73.47 ± 13.82 ($p \le 0.05$) of IL-6 and 130.23 ± 9.50 pg/ml ($p \le 0.05$) and 172.64 ± 14.97 pg/ml ($p \le 0.01$) IL-8 respectively. At later time-points there was an increase in these cytokines, such that following 4 h stimulation with LPS there was a marked up-regulation of IL-6 and IL-8 with 112.0 ± 11.3 pg/ml ($p \le 0.05$) and 219.45 ± 12.22 pg/ml for IL-6 and IL-8 respectively. Incubation of chondrocytes with TNF-α (60.0 pg/ml) and LPS (1.0 µg/ml) for 6 h caused 117.1 ± 26.1 pg/ml and 135.99 ± 20.13 pg/ml release of IL-6, for the two inflammogens respectively, and 205.92 ± 27.10 pg/ml and 223.74 ± 15.33 pg/ml release of IL-8, respectively ($p \le 0.05$).
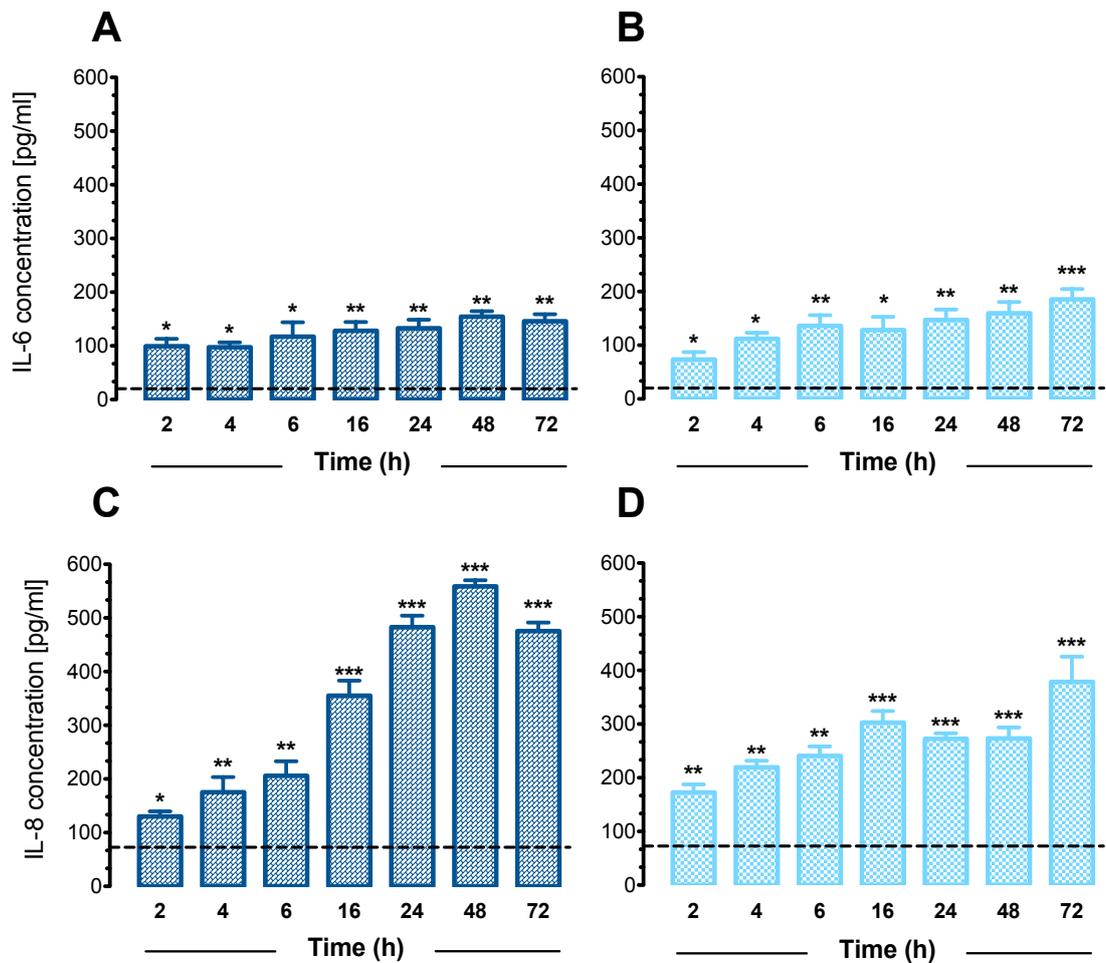
Figure 3.9. Effect of TNF-α and LPS on IL-6 and IL-8 release in C-20/A4 cells over 0-72 h period.

C-20/A4 chondrocytes were stimulated with TNF-α (60.0 pg/ml; dark blue bars) or LPS (1.0 μg/ml; light blue bars) and cell-free supernatants collected at 0 – 72 h. IL-6 (Panel A, B) and IL-8 (Panel C, D) were then determined by ELISA. Data are presented as Mean ± SEM of n=7 independent experiments repeated in triplicate, *$p \leq$ 0.05, **$p \leq$ 0.01, ***$p \leq$ 0.001 vs. Time 0 (represented by a dotted line).

3.1.3 Effect of TNF-α and LPS on *MMP1, MMP3* and *MMP13* expression in C20/A4 chondrocytes.

OA is increasingly characterised by release of pro-inflammatory cytokines and other mediators of the host inflammatory response and cartilage degradation initiated by MMPs. Generally, collagenase expression induced by TNF-α is thought to be NFκB-dependent, a pathway also used by TLRs (Zhang *et al.,* 2008). Initially, the effects of TNF-α and LPS (TLR4 ligand) were investigated on *MMP1*, *MMP3* and *MMP13* expression by C20/A4 chondrocytes. The effect of TNF-α (20.0 – 80.0 pg/ml) and LPS (0.1 – 1.0 μg/ml) on MMPs levels were at first evaluated following 6 h stimulation of C-20/A4 chondrocytes and the total RNA extracted from stimulated and non-stimulated cells was analysed for transcription of these genes by RT-PCR.

LPS and TNF-α caused increases in *MMP1* (Figure 3.10 A) and *MMP13* (Figure 3.11 A) and decreased expression of *MMP3* in C-20/A4 cells at 6 h. (Figure 3.12 A). TNF-α (20.0 – 80.0 pg/ml) caused significant increases in *MMP1* expression (Figure 3.10 B), with 20.0 pg/ml causing 1.5-fold increase compared to basal (0.21 ± 0.02 au to 0.33 ± 0.04 au, p≤ 0.05). Increased concentrations of TNF-α (40.0 and 60.0 pg/ml) led to a plateau effect of 8-fold up-regulation of *MMP1* with expression levels of 1.66 ± 0.22 au and 1.71 ± 0.11 au respectively (p≤ 0.01 for both concentrations), whilst 80.0 pg/ml of TNF-α caused a slightly less profound but similar increase to 1.52 ± 0.23 au representing 7-fold increase in expression (*p*≤ 0.01). LPS stimulation caused a bell-shaped response with a significant increase in *MMP1* expression at 0.1 μg/ml (8-fold increase; 1.78 ± 0.21 au, p<0.01) and at 1.0 μg/ml (7-fold increase; 1.47 ± 0.1 au, p≤ 0.01), with a maximal 12-fold up-regulation detected following stimulation with 0.3 μg/ml LPS (2.18 ± 0.3 au, p≤ 0.01; Figure 3.10 C).

Densitometric analysis of *MMP13* expression showed a concentration dependent increase in expression (Figure 3.11 A), whilst no detection was observed in non-stimulated C-20/A4 chondrocytes. TNF-α (20.0 and 40.0 pg/ml) caused an increase in *MMP13* expression to 0.057 ± 0.03 au and 0.1 ± 0.02 au, respectively (p≤ 0.05). TNF-α 60.0 pg/ml and 80.0 pg/ml caused 0.23 ± 0.01 ± 0.002 au and

0.34 ± 0.07 au respectively ($p \leq$ 0.001; Figure 3.11 B). Similarly, LPS (0.1 – 1.0 μg/ml) at all concentrations led to significant increases in *MMP13* expression levels (Figure 3.11 C), with 0.1 μg/ml causing 0.13 ± 0.003 au and higher concentrations causing 0.205 ± 0.002 au and 0.2 ± 0.03 au ($p \leq$ 0.001 both) expression for 0.3 μg/ml and 1.0 μg/ml respectively.

MMP3 was then evaluated as it has been shown to be highly expressed in healthy hip cartilage, and significantly reduced in osteoarthritic hip cartilage (Kevorkian *et al*., 2004). Both TNF-α and LPS significantly reduced *MMP3* levels (Figure 3.12 A). TNF-α stimulation caused a bell-shaped inhibition in *MMP3* expression with 60.0 pg/ml, with a 46% down-regulation of the protease expression from 0.580 ± 0.051 au to 0.31 ± 0.05 au ($p \leq$ 0.05; Figure 3.12 B). Treatment with LPS caused a concentration dependent decrease in *MMP3* expression with 1.0 μg/ml causing a complete inhibition in expression compared to untreated controls ($p \leq$ 0.001). Lower concentrations of LPS caused significant inhibition with 0.1 μg/ml and 0.3 μg/ml leading to 64% and 77% down-regulation of *MMP3* expression (0.21 ± 0.04 and 0.13 ± 0.06 au, $p \leq$ 0.01) compared to control cultures (Figure 3.12 C).

Following identification of *MMP1, MMP3* and *MMP13* mRNA expression at 6 h, the effect of TNF-α in a time-dependent manner was investigated. C-20/A4 chondrocytes were stimulated with TNF-α (60.0 pg/ml) for 0 – 48 h. *MMP1* was markedly increased in a time-dependent manner (Figure 3.13 A). Unlike *IL6* and *IL8* expression, which peaked at 2 h post-stimulation, TNF-α stimulation of *MMP1* did not cause significant change in expression at this time point (0.31 ± 0.04 au compared to untreated 0.22 ± 0.03 au). However, at 6 h there was a marked 8-fold increase to 1.71 ± 0.11 au ($p < 0.001$). The expression of *MMP1* continued to increase over the time course to 2.12 ± 0.22 au ($p \leq$ 0.001) at 24 h and was reduced at 48 h (1.72 ± 0.23 au, 8-fold; $p \leq$ 0.001; Figure 3.13 B).

Similarly, *MMP13* expression was unchanged following 2 h stimulation with TNF-α (0.021 ± 0.022 au; $p > 0.05$); however, expression was significantly increased at 6 h with a 11.5-fold increase in expression compared to 2 h ($p < 0.001$; Figure 3.13 D). Increasing the length of the incubation time caused further increases in

expression of *MMP13* with 0.29 ± 0.03 au and 0.34 ± 0.07 au at 24 h and 48 h post-incubation respectively.

Treatment of C-20/A4 with TNF-α (60.0 pg/ml) caused a significant reduction of *MMP3.* It was significantly reduced by 55% down to 0.26 ± 0.05 au ($p \leq 0.01$) at 2 h, by 36% down to 0.37 ± 0.42 au ($p \leq 0.05$) and at 24 h the expression of *MMP3* was down-regulated to 0.31 ± 0.05 au, a 46% decrease ($p \leq 0.05$) as compared to untreated controls (0.580 ± 0.051 au). At 48 h post stimulation, there was a slight non-significant inhibition of *MMP3* expression (Figure 3.13 C).
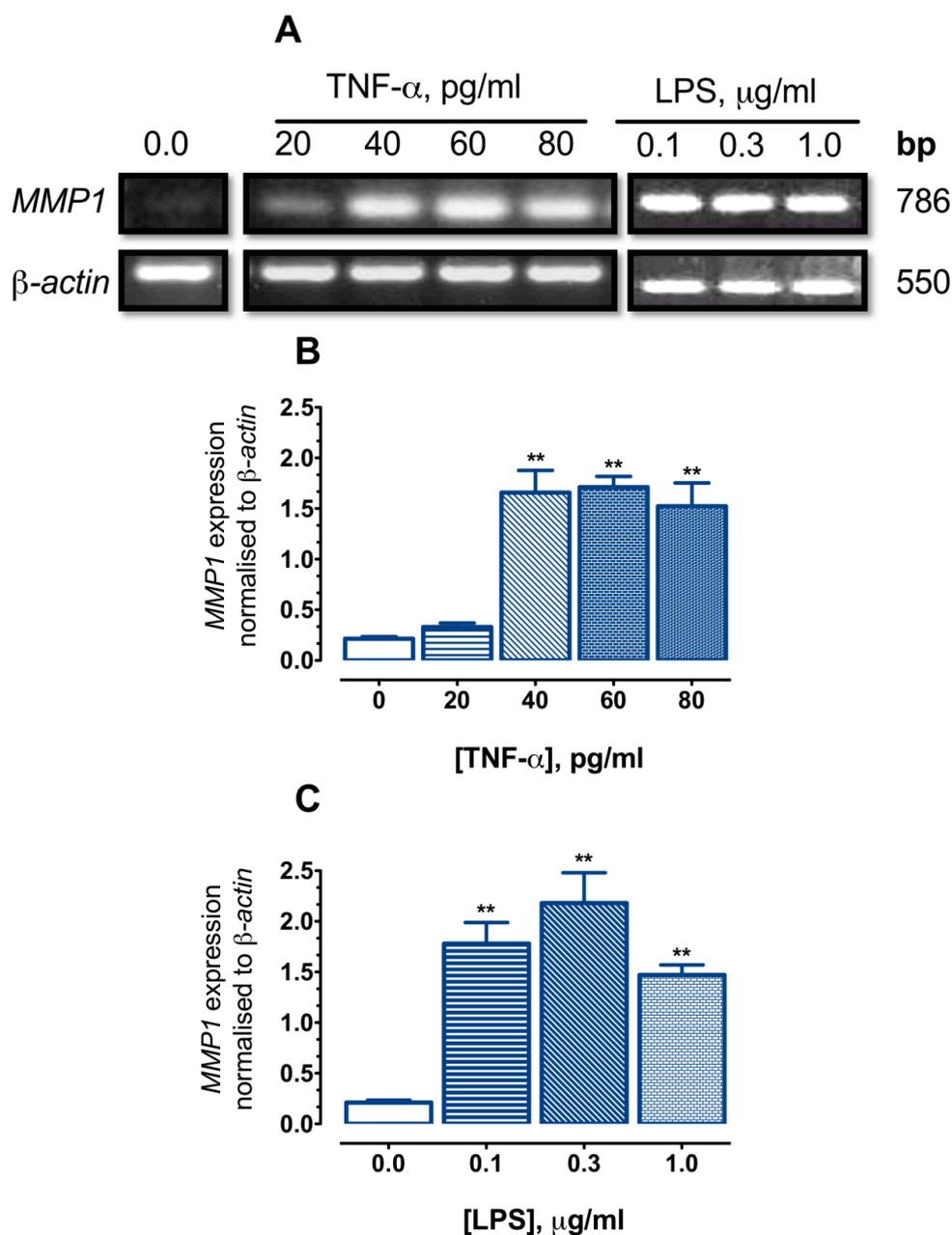
Figure 3.10. Densitometric quantification of *MMP1* expression in C-20/A4 cells treated with TNF-α (20.0 – 80.0 pg/ml) and LPS (0.1 – 1.0 μg/ml) for 6 h.

C-20/A4 chondrocytes were stimulated with TNF-α (20.0 – 80.0 pg/ml) or LPS (0.1 – 1.0 μg/ml) at time 0 and total RNA extracted 6 h post stimulation. PCR amplification with the respective primers for *MMP1* was used to detect and quantify gene expression on 2 % agarose gels in triplicates with *β-actin* used as internal control (Panel A). Comparison of densitometrically quantified *MMP1* expression by TNF-α (Panel B) and LPS (Panel C) shown in arbitrary units, each value normalized to the respective *β-actin* expression. Data are presented as Mean ± SEM of four independent experiments **$p \leq 0.01$.
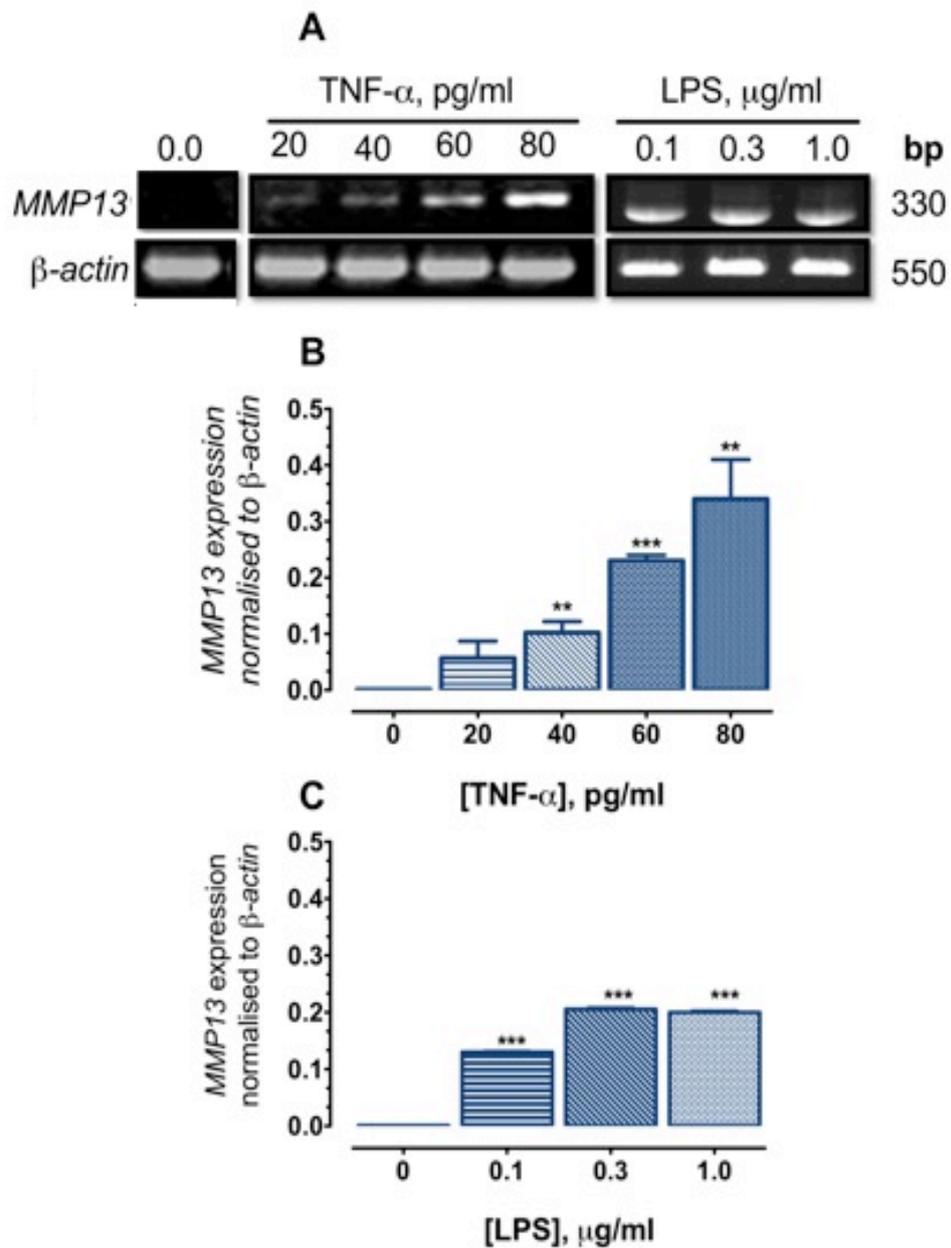
Figure 3.11. Densitometric quantification of *MMP13* expression in C-20/A4 cells treated with TNF-α (20.0 – 80.0 pg/ml) and LPS (0.1 – 1.0 µg/ml) for 6 h.

C-20/A4 chondrocytes were stimulated with TNF-α (20.0 – 80.0 pg/ml) or LPS (0.1 – 1.0 µg/ml) at time 0 and total RNA extracted 6 h post stimulation. PCR amplification with the respective primers for *MMP13* was used to detect and quantify gene expression on 2 % agarose gels in triplicates with *β-actin* used as internal control (Panel A). Comparison of densitometrically quantified *MMP13* expression by TNF-α (Panel B) and LPS (Panel C) shown in arbitrary units, each value normalized to the respective *β-actin* expression. Data are presented as Mean ± SEM of four independent experiments **$p \leq$ 0.01, ***$p \leq$ 0.001.

A

B

C
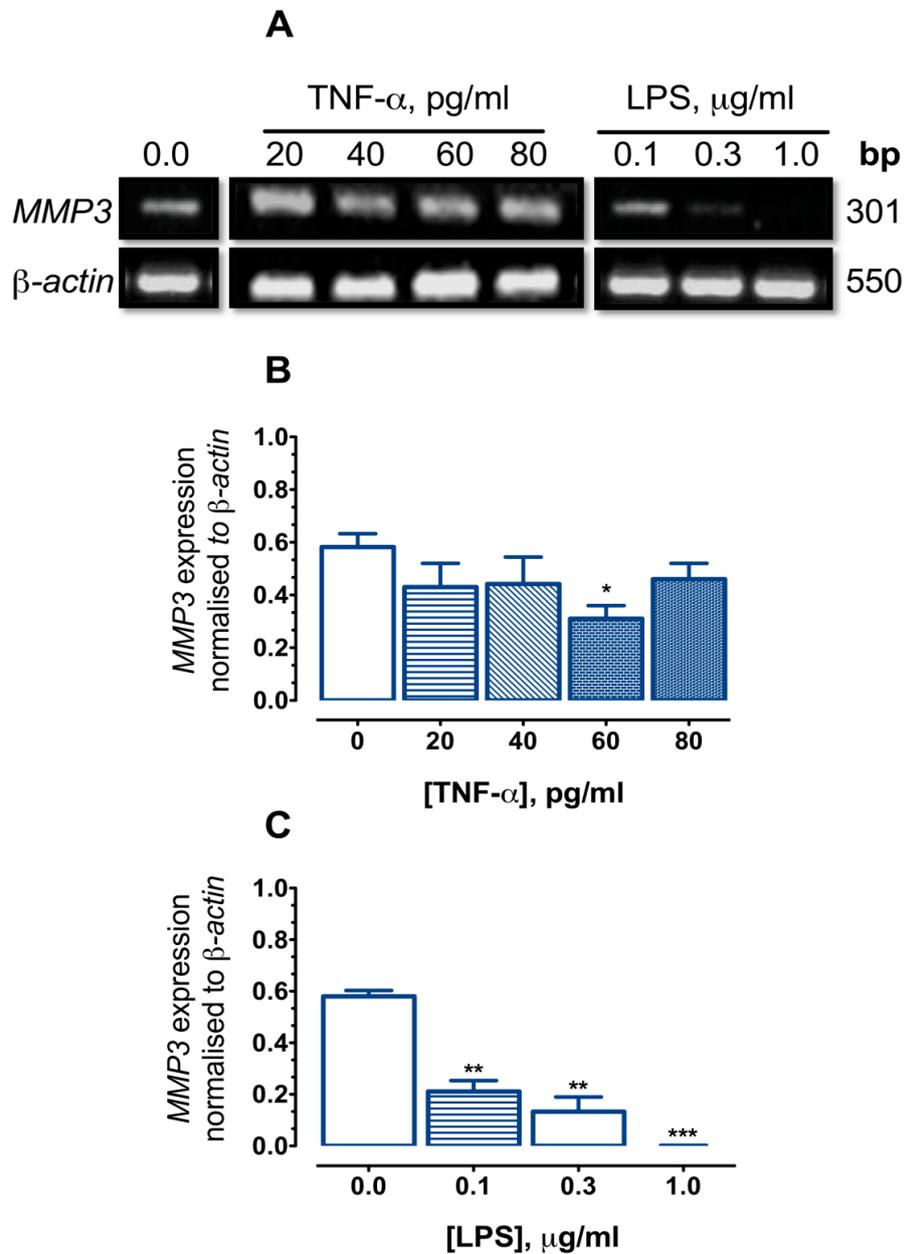
Figure 3.12. Densitometric quantification of *MMP3* expression in C-20/A4 cells treated with TNF-α (20.0 – 80.0 pg/ml) and LPS (0.1 – 1.0 μg/ml) for 6 h.

C-20/A4 chondrocytes were stimulated with TNF-α (20.0 – 80.0 pg/ml) or LPS (0.1 – 1.0 μg/ml) at time 0 and total RNA extracted 6 h post stimulation. PCR amplification with the respective primers for MMP3 was used to detect and quantify gene expression on 2 % agarose gels in triplicates with β-actin used as internal control (Panel A). Comparison of densitometrically quantified *MMP3* expression by TNF-α (Panel B) and LPS (Panel C) shown in arbitrary units, each value normalized to the respective *β-actin* expression. Data are presented as Mean ± SEM of four independent experiments, **$p \leq$ 0.01, ***$p \leq$ 0.001.
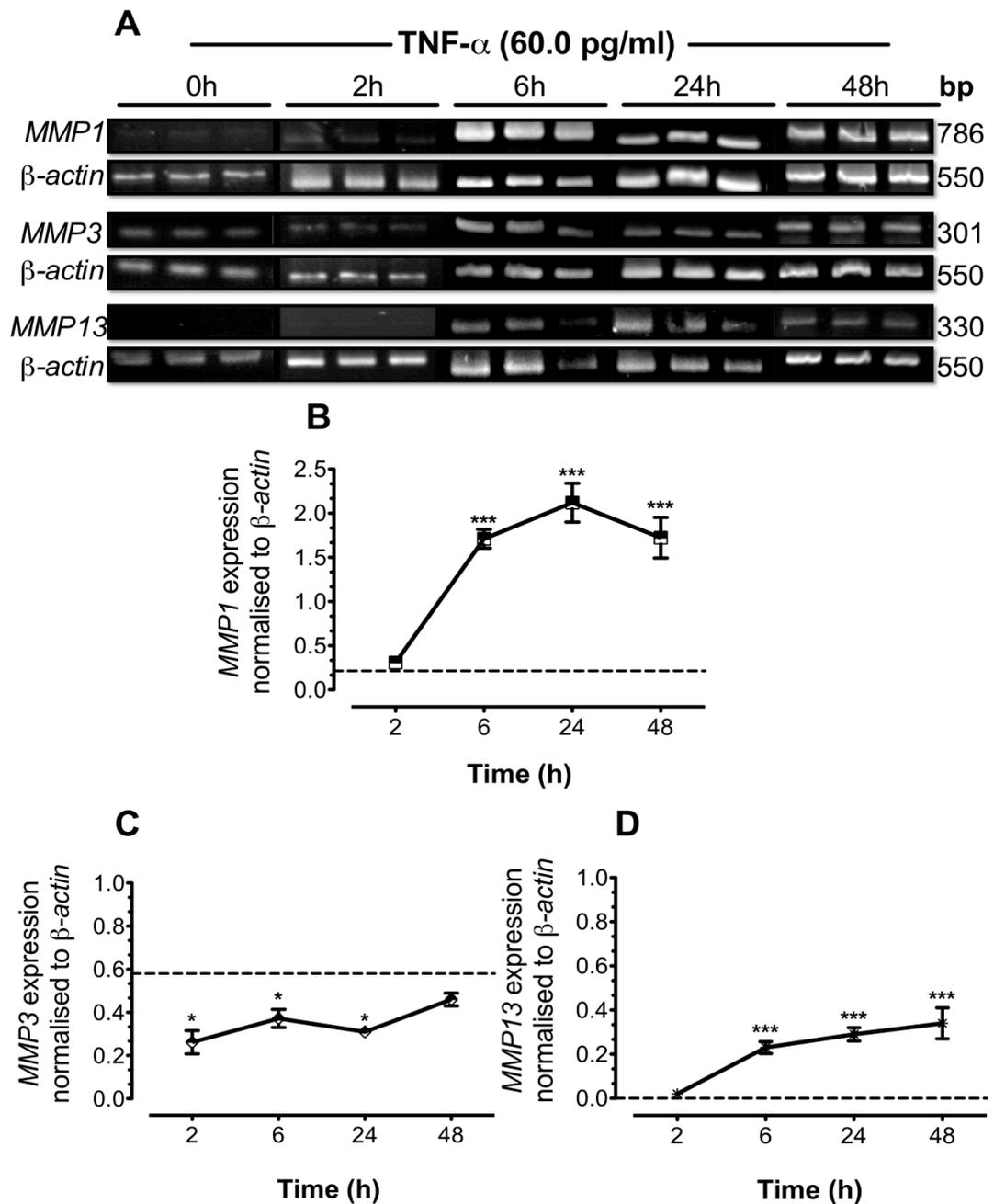
Figure 3.13. Densitometric quantification of *MMP1*, *MMP3* and *MMP13* expression in C-20/A4 cells treated with TNF-α (60.0 pg/ml) for 0-48 h.

C-20/A4 chondrocytes were stimulated with TNF-α (20.0 – 80.0 pg/ml) at time 0 and total RNA extracted 0-48 h post stimulation. PCR amplification with the respective primers for *MMP1*, *MMP3* and *MMP13* were used to detect and quantify gene expression on 2 % agarose gels in triplicates with *β-actin* used as internal control (Panel A). Comparison of densitometrically quantified *MMP1* (Panel B), *MMP3* (Panel C) and *MMP13* (Panel D) expression by TNF-α shown in arbitrary units, each value normalized to the respective *β-actin* expression. Dotted line represents basal expression levels. Data are presented as Mean ± SEM of four independent experiments *$p ≤ 0.05$, ***$p ≤ 0.001$.

3.1.4 Effect of TNF-α on *COL2A1* and *COL1A1* expression.

In healthy articular cartilage, chondrocytes are actively maintaining the steady-state expression of collagens and proteoglycans. Articular chondrocytes are sensitive to various growth factors and cytokines, which either enhance or reduce the synthesis of type II collagen, a marker of normal function of articular chondrocytes (Goldring *et al.,* 1994). In osteoarthritis, cytokines including IL-1β, IL-6, IL-8, and TNF-α, produced by osteoarthritic chondrocytes significantly up-regulate matrix metalloproteinases (MMPs) gene expression, and decrease the synthesis of tissue specific macromolecules, such as collagen II, therefore inhibiting the chondrocyte's compensatory synthesis pathways required to restore the integrity of the degraded extracellular matrix (Pelletier *et al*., 1991, Goldring *et al*., 1994b, Shlopov *et al*., 1997, Shlopov *et al*., 2000, Goldring *et al*., 2011).

Human C-20/A4 chondrocytes were found to respond to TNF-α in a time and concentration-dependent manner, by producing large amounts of the above-mentioned pro-inflammatory cytokines and significantly up-regulating collagenases 1 and 3. Next, in order to further investigate whether stimulation with TNF-α induces conditions resembling the events observed in OA chondrocytes, we investigated its effect on *COL1A1*, *COL2A1* expression and additionally calculated the *COL2A1*:*COL1A1 ratio* to study the effect of TNF-α on the chondrocytic phenotype of the C-20/A4 cells. The results showed that expression of *COL2A1*, a marker for normal function of articular chondrocytes, was significantly down-regulated after TNF-α (60.0 pg/ml) treatment, in comparison to unstimulated cultures of C-20/A4 chondrocytes. The inflammogen caused a 66.7 % reduction in mRNA expression of collagen type II from 0.754 $\pm$ 0.08 au to 0.24 $\pm$ 0.012 au ($p \leq$ 0.01) and 49 % reduction in *COL1A1* expression from 2.25 $\pm$ 0.16 au to 1.121 $\pm$ 0.05 au ($p \leq$ 0.01), as calculated following densitometric analysis (Figure 3.14 B) of the PCR product bands with the correct size (Figure 3.14 A).

Despite the large drop in overall collagen production, there was only slight, insignificant change in the indicator of differentiation *COL2A1*:*COL1A1* ratio, as compared to untreated C-20/A4 chondrocytes, indicated in Figure 3.14 C.
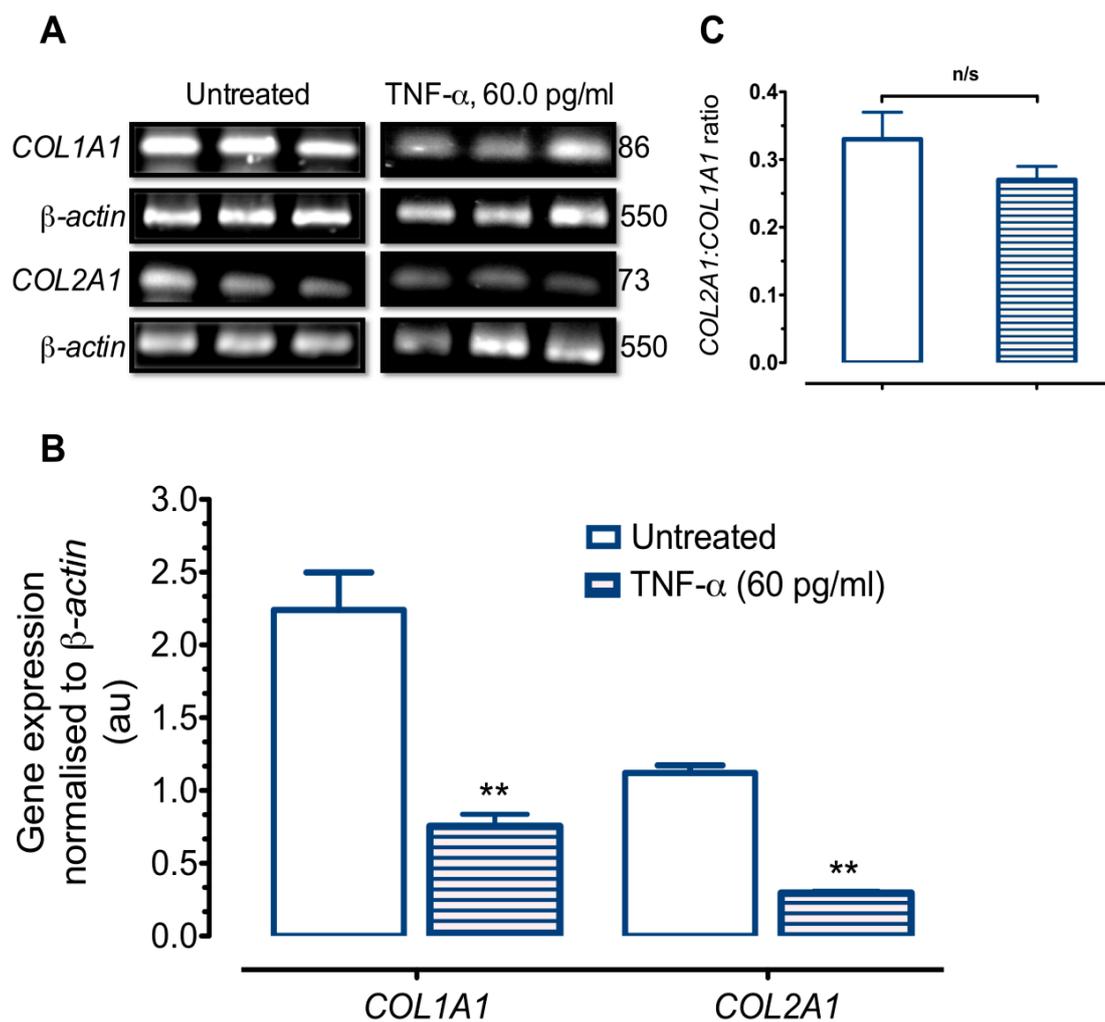
Figure 3.14 Quantification of the effect of TNF-α on *COL1A1* and *COL2A1* expression.

C-20/A4 chondrocytes were treated with TNF-α (60.0 pg/ml) and total RNA extracted 6 h post stimulation. PCR amplification with the respective primers for *COL1A1* and *COL2A1* was used to detect and quantify gene expression on 2 % agarose gels in triplicates with *β-actin* used as internal control (Panel A). Comparison of densitometrically quantified *COL1A1* and *COL2A1* expression (Panel B) is shown in arbitrary units (au), each value normalized to the respective *β-actin* expression. Comparison of *COL2A1:COL1A1* ratio prior and after stimulation with TNF-α is shown in Panel C. Data are presented as Mean ± SEM of 3 independent experiments **$p \leq 0.01$.

3.2 Effect of classical anti-inflammatory drugs on inflammatory mediator release from C-20/A4 chondrocytes

To determine whether inflammatory pathways were modulated in the presence of classical anti-inflammatory drugs, the effect of the non-steroidal anti-inflammatory drug indomethacin and glucocorticoid dexamethasone on stimulated chondrocytes was evaluated. Indomethacin is an indol derivative that's a non-selective COX-1 and COX-2 inhibitor with anti-inflammatory, analgesic, and antipyretic effects (Hart and Boardman, 1963). Dexamethasone is a potent synthetic member of the glucocorticoid class of steroid drugs. It acts as an anti-inflammatory and immunosuppressant drug and is 20-30 times more potent than the naturally occurring cortisol and 4-5 times more potent than prednisone (Vayssiere *et al*., 1997). Dexamethasone and indomethacin are used to treat many inflammatory and autoimmune diseases including osteoarthritis and rheumatoid arthritis.

3.2.1 Effect of indomethacin on IL-6 and IL-8 mRNA and protein in C-20/A4 chondrocytes.

C-20/A4 cell cultures were treated with 1.0 μM indomethacin for 30 min prior to 6 h incubation with TNF-α (60.0 pg/ml). Total RNA was extracted and PCR analysis performed using specific primers for the amplification of *IL-6* and *IL-8* (Table. 2.9). A significant decrease was observed in *IL-6* and *IL-8* expression following treatment with indomethacin (1.0 μM; Figure 3.15 A) with a significant 61.4 %, ($p \leq$ 0.001) and 78.6 % ($p \leq$ 0.001) down-regulation of *IL-6* and *IL-8* respectively as determined by densitometric quantification compared to β-*actin* (Figure 3.15 B).

Subsequently, its effect on IL-6 and IL-8 protein levels was determined. C-20/A4 chondrocytes were pre-treated with 1.0 μM indomethacin for 30 min prior to stimulation with TNF-α (60.0 pg/ml) and cell-free supernatants collected and analysed for IL-6 and IL-8 by ELISA. Indomethacin completely abrogated the release of the pro-inflammatory cytokines IL-6 and IL-8 bringing it down to basal levels (Figure 3.16 A and B).
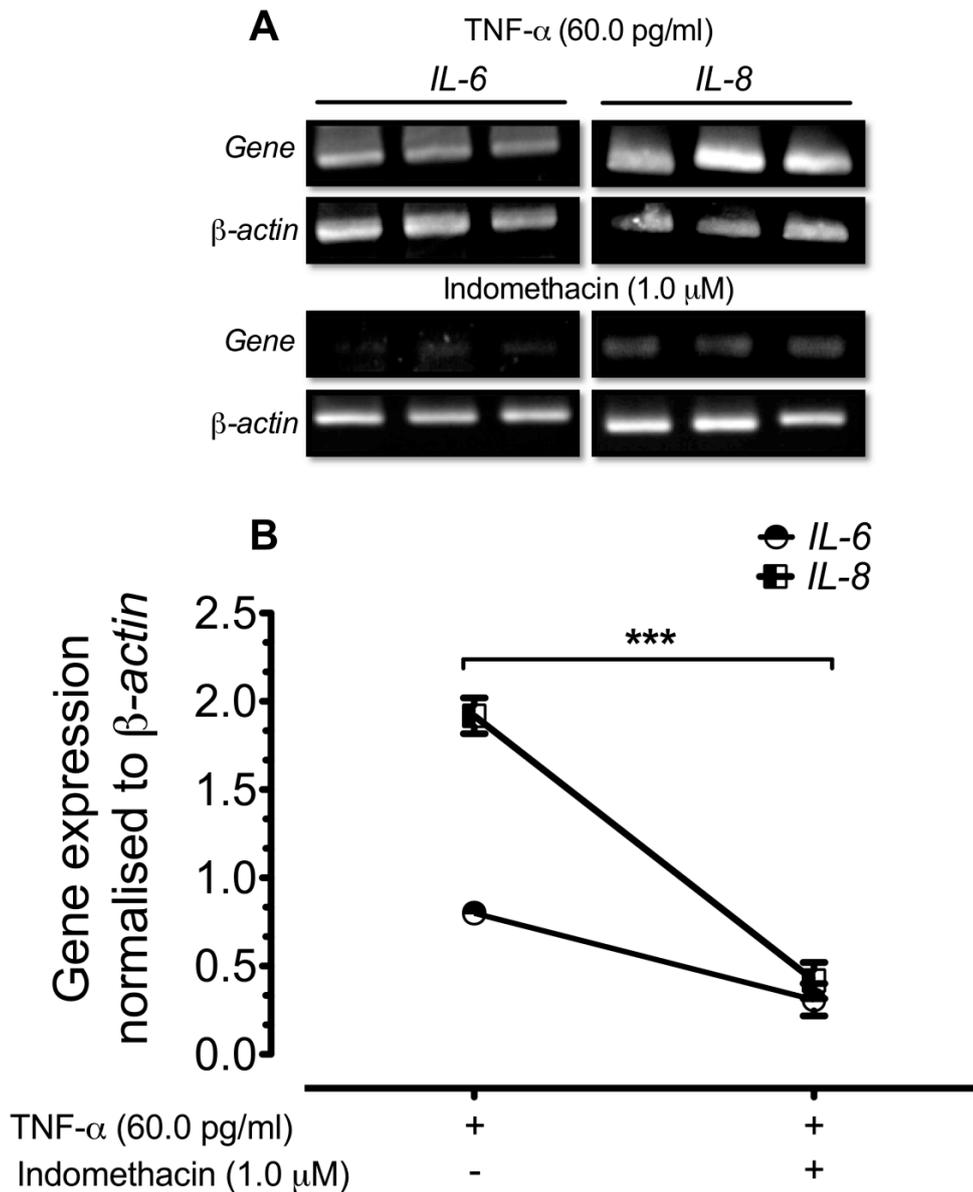
Figure 3.15. Densitometric quantification of the effect of indomethacin on TNF-α-mediated *IL-6* and *IL-8* gene expression.

C-20/A4 chondrocytes were pre-treated for 30 min with PBS or indomethacin (1.0 μM) prior to stimulation with TNF-α (60.0 pg/ml) and total RNA extracted 6 h post stimulation. PCR amplification with the respective primers for *IL6* and *IL8* was used to detect and quantify gene expression on 2 % agarose gels in triplicates with β-actin used as internal control (Panel A). Comparison of densitometrically quantified *IL6* and *IL8* expression by TNF-α (Panel B) shown in arbitrary units, each value normalized to the respective *β-actin* expression. Data are presented as Mean± SEM of four independent experiments ***$p \leq$ 0.001.

Figure 3.16. Effect of Indomethacin on TNF-α-mediated IL-6 and IL-8 release.

C-20/A4 chondrocytes were pre-treated for 30 min with PBS or Indomethacin (1.0 μM) prior to stimulation with TNF-α (60.0 pg/ml) and cell-free supernatants collected at 6 h post stimulation. IL-6 (Panel A) and IL-8 (Panel B) expression by TNF-α were then determined. Data are presented as Mean± SEM of n=4 independent experiments repeated in triplicate, **$p \leq$ 0.01, ***$p \leq$ 0.001.

3.2.2 Effect of dexamethasone on IL-6 and IL-8 mRNA and protein on C20/A4 chondrocyte.

In order to investigate the effect of dexamethasone on the expression of pro-inflammatory cytokines *IL6* and *IL8* in TNF-α treated C-20/A4 chondrocytes, cells were pre-treated with 1.0 µM dexamethasone for 30 mins before stimulation with TNF-α (60.0 pg/ml) and incubated for 6 h. PCR analysis was performed using specific primers for the amplification of *IL6* and *IL8* (Table. 2.9). Pre-treatment of C-20/A4 cells with 1.0 µM dexamethasone led to a significant 72.6 % down-regulation of *IL6* and *IL8* by 83 % compared to control (Figure 3.17). This effect at the mRNA level was confirmed at the protein level with a complete abrogation of TNF-α induced *IL6* and *IL8* release (Figure 3.18).
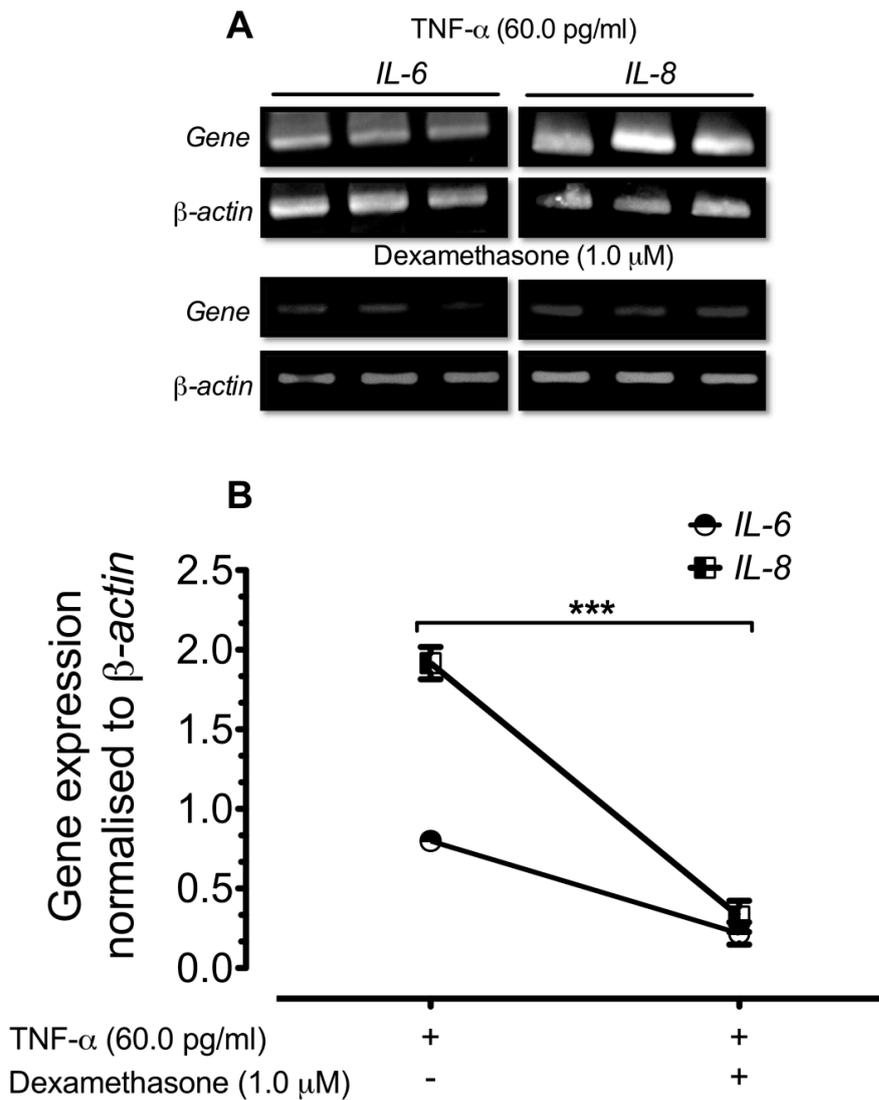
Figure 3.17. Densitometric quantification of the effect of dexamethasone on TNF-α-mediated *IL6* and *IL8* gene expression.

C-20/A4 chondrocytes were pre-treated for 30 min with PBS or dexamethasone (1.0 μM) prior to stimulation with TNF-α (60.0 pg/ml) and total RNA extracted 6 h post stimulation. PCR amplification with the respective primers for *IL6* and *IL8* was used to detect and quantify gene expression on 2% agarose gels in triplicates with β-actin used as internal control (Panel A). Comparison of densitometrically quantified *IL6* and *IL8* expression by TNF-α (Panel B) shown in arbitrary units, each value normalized to the respective *β-actin* expression. Data is presented as Mean ± SEM of n=4 independent experiments ***$p \leq$ 0.001.

**Figure 3.18. Effect of Dexamethasone on TNF-α-mediated IL-6 and IL-8 protein release.**

C-20/A4 chondrocytes were pre-treated for 30 min with PBS or dexamethasone (1.0 μM) prior to stimulation with TNF-α (60.0 pg/ml) and cell-free supernatants collected at 6 h post stimulation. TNF-α stimulated IL-6 (Panel A) and IL-8 (Panel B) production was then determined. Data are presented as Mean ± SEM of n=4 independent experiments repeated in triplicate, *p≤ 0.05, **p≤ 0.01, ***p≤ 0.001.

3.2.3 Effect of indomethacin on *MMP1, MMP3* and *MMP13* gene expression.

The effect of indomethacin on the regulation of *MMP1, MMP3* and *MMP13* gene expression in non-stimulated and TNF-α stimulated C-20/A4 chondrocytes was investigated (Figure 3.19)

C-20/A4 cells were pre-treated with 1.0 µM Indomethacin for 30 min prior to stimulation with TNF-α (60.0 pg/ml) for 6 h. Total RNA was extracted and PCR analysis performed using specific primers for the amplification of *MMP1, MMP3* and *MMP13* (Table. 2.9; Chapter 2). Following gel electrophoresis of the PCR reactions on 2 % agarose gels, the correct bands (Figure 3.19 A) were quantified densitometrically and a significant decrease in the proteinases expression ($p \leq$ 0.01) was detected with a >75 % inhibition of the interstitial collagenase *MMP1*, 67% reduction in *MMP13* and a 49 % down-regulation of *MMP3* expression compared to TNF-α stimulated controls (Figure 3.19 B).

3.2.4 Effect of dexamethasone on *MMP1, MMP3* and *MMP13* gene expression.

Following identification of the anti-cytokine properties of dexamethasone, its effect on the expression of *MMP1, MMP3* and *MMP13* in non-stimulated and TNF-α-treated C-20/A4 chondrocytes was investigated (Figure 3.20).

C-20/A4 cells were pre-treated with 1.0 µM dexamethasone for 30 minutes prior to stimulation with TNF-α (60.0 pg/ml) for 6 h. Total RNA was extracted and PCR analysis performed using specific primers for the amplification of *MMP1, MMP3* and *MMP13* (Table. 2.9,). A significant decrease in the proteinases expression ($p \leq$ 0.01) was detected with a >75 % inhibition of *MMP1*, 42 % reduction in *MMP13* and a 49 % down-regulation of *MMP3* expression compared to TNF-α stimulated controls (Figure 3.20).
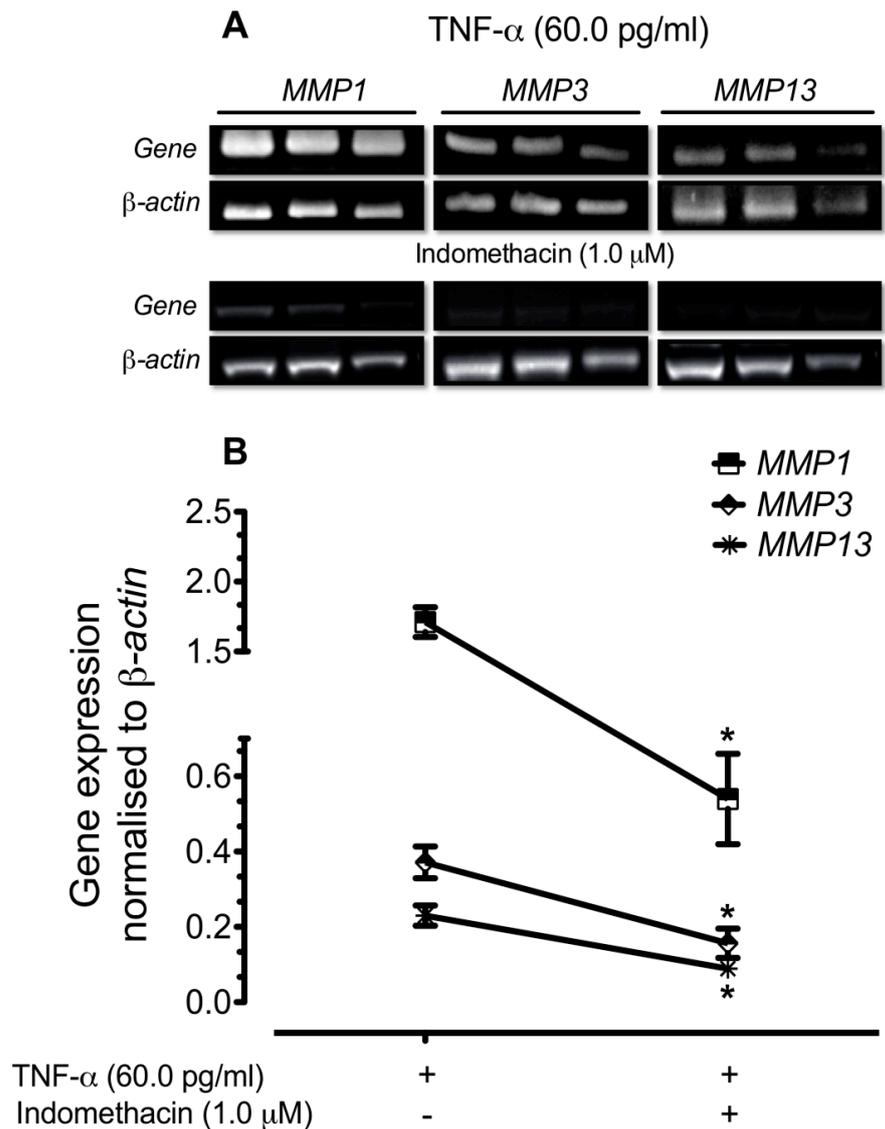
Figure 3.19. Effect of Indomethacin on TNF-α-mediated *MMP1, MMP3* and *MMP13* expression.

C-20/A4 chondrocytes were pre-treated for 30 min with PBS or indomethacin (1.0 μM) prior to stimulation with TNF-α (60.0 pg/ml) at time 0 and total RNA extracted 6 h post stimulation. PCR amplification with the respective primers for *MMP1*, *MMP3* and *MMP13* were used to detect and quantify gene expression on 2 % agarose gels in triplicates with β-actin used as internal control (Panel A). Densitometrically quantified *MMP1*, *MMP3* and *MMP13* expression by TNF-α alone or in presence of Indomethacin shown in arbitrary units, each value normalized to the respective *β-actin* expression (Panel B). Data are presented as Mean ± SEM of n=4 independent experiments *$p \leq$ 0.05.
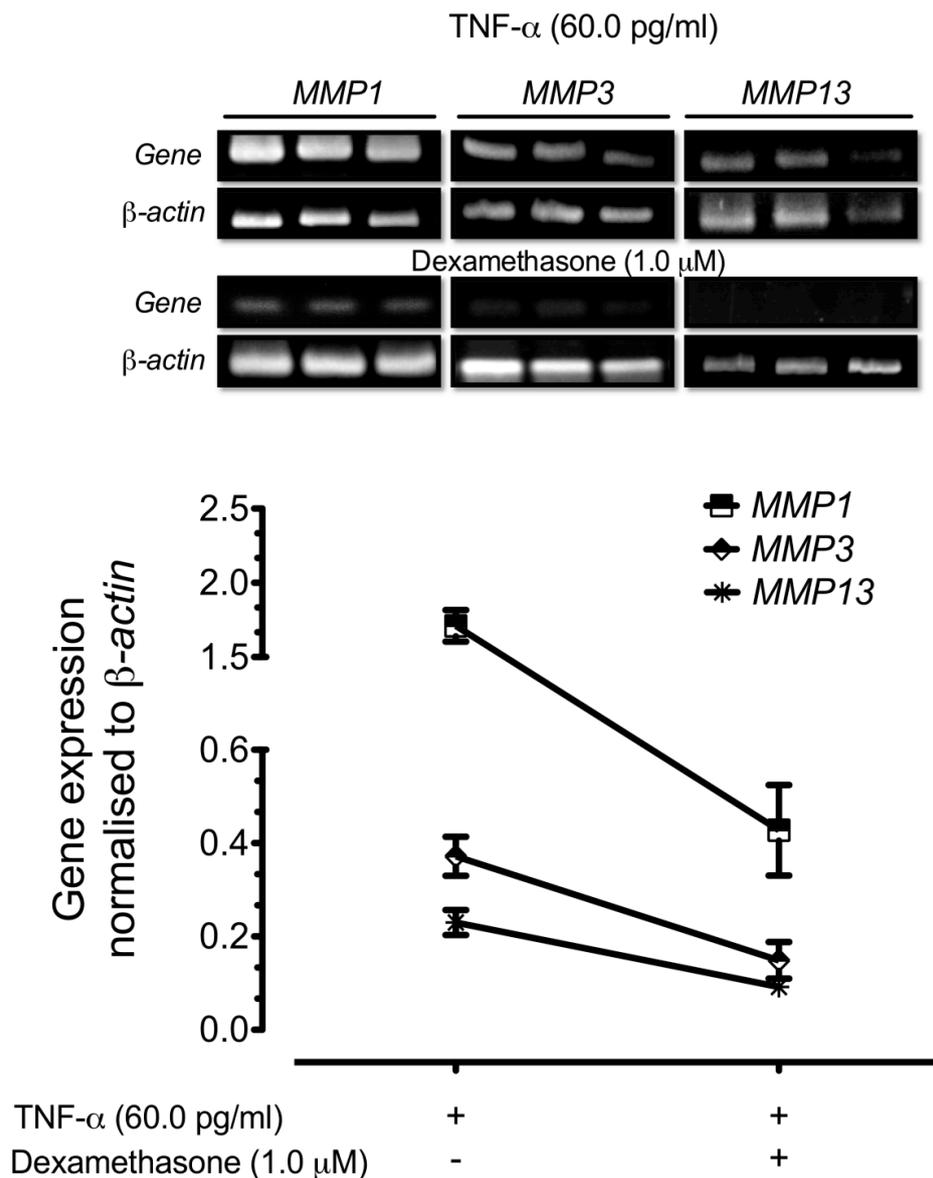
Figure 3.20. Effect of Dexamethasone on TNF-α-mediated *MMP1, MMP3* and *MMP13* expression.

C-20/A4 chondrocytes were pre-treated for 30 min with PBS or dexamethasone (1.0 μM) prior to stimulation with TNF-α (60.0 pg/ml) at time 0 and total RNA extracted 6 h post stimulation. PCR amplification with the respective primers for *MMP1*, *MMP3* and *MMP13* were used to detect and quantify gene expression on 2 % agarose gels in triplicates with *β-actin* used as internal control (Panel A). Densitometrically quantified *MMP1*, *MMP3* and *MMP13* expression by TNF-α alone or in presence of dexamethasone shown in arbitrary units, each value normalized to the respective *β-actin* expression (Panel B). Data are presented as Mean ± SEM of n=4 independent experiments *$p \leq 0.05$.

3.3 Effect of melanocortin peptides treatment on C-20/A4 activation.

3.3.1 Expression of MC_1, MC_3 and MC_5 mRNA and protein on C-20/A4 chondrocytic cells.

Melanocortin peptides (e.g. α-MSH) have been shown to possess anti-pyretic and anti-inflammatory functions *via* activation of a family of seven transmembrane receptors termed melanocortin receptors of which five have been identified (MC_{1-5}, Getting *et al.*, 2009). Expression of MC_1, MC_3 and MC_5 by C20/A4 chondrocytes was determined.

Total RNA was extracted from the cells and reverse-transcribed into cDNA via RT-PCR. PCR amplification reactions were used to assay for MC_1, MC_3 and MC_5 expression. MC_1 and MC_3 were highly expressed on C-20/A4 chondrocytes, with detection of specific bands corresponding to the expected size 493 bp and 820 bp respectively. Very slight MC_5 expression was also detected at the expected size of 340 bp. All samples were run alongside β-*actin* as an internal control (Figure 3.21).

Following identification of MC_1 and MC_3 mRNA, protein levels of these receptors were determined by employing Western Blotting. Protein extracts were prepared and examined for MC_1 and MC_3 protein expression. Western Blotting confirmed the presence of both of the melanocortin receptors in human C-20/A4 chondrocytes at the expected size of 35 and 40 kDa for MC_1 and MC_3 respectively. C-20/A4 cells expressed lower levels of MC_3 compared to MC_1 receptor after both were compared and normalized to the internal control α-Tubulin (Figure 3.22).
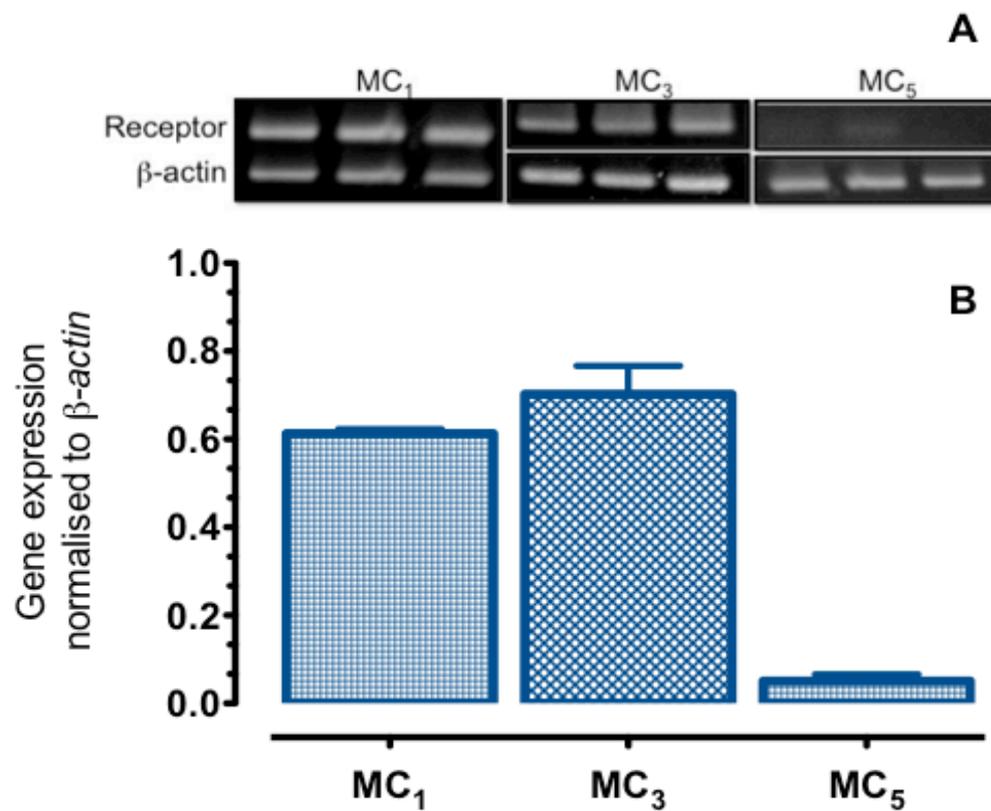
Figure 3.21. Endogenous expression of *MC₁*, *MC₃* and *MC₅* by C-20/A4 chondrocytic cell-line.

C-20/A4 chondrocytes were grown to confluence and RNA extracted. PCR amplification with the respective primers for MC_1, MC_3 and MC_5 were used to detect and quantify gene expression on 2 % agarose gels in triplicates with *β-actin* used as internal control. Densitometrically quantified MC_1, MC_3 and MC_5 shown in arbitrary units, each value normalized to the respective *β-actin* expression. Data are presented as Mean ± SEM of 5 independent experiments.
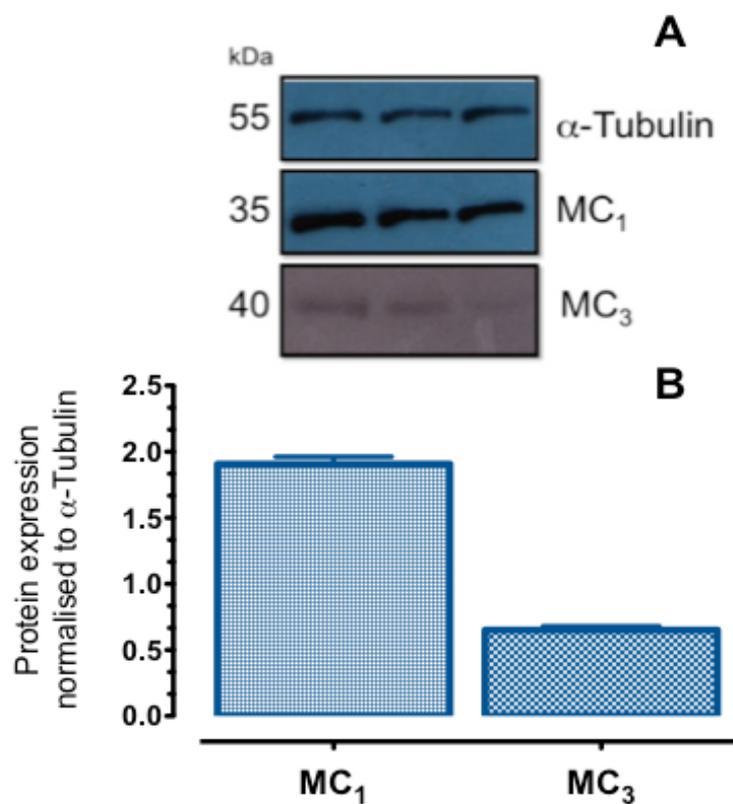
Figure 3.22. Detection of MC$_1$ and MC$_3$ protein in human C-20/A4 chondrocytic cells.

Protein detection was performed using mouse anti-α-Tubulin mAb, rabbit anti-MC$_1$ mAb and rabbit anti-MC$_3$ mAb (1:2000). Bands with sizes corresponding to MC$_1$ (35 kDa), MC$_3$ (40 kDa) and α-tubulin (55 kDa) were detected and melanocortins receptor expression confirmed in C-20/A4 cells. The image is a representative of 3 individual experiments.

3.3.2 cAMP accumulation in C-20/A4 chondrocytes following melanocortin peptide stimulation.

Following identification of MC_1, MC_3 and MC_5 mRNA and MC_1, MC_3 protein, a panel of melanocortin peptides was evaluated to ascertain receptor functionality by determining intracellular cAMP accumulation within the C20/A4 chondrocytes. C-20/A4 cells were treated with α-MSH, D[TRP]^8-γ-MSH, SHU9119, PG910, PG911 or a direct adenylate cyclase stimulator, forskolin, used as positive control in all cases. Forskolin (3.0 μM) increased the intracellular cAMP level to 2227.44 ± 74.42 fmol/well, representing 9-fold increase over PBS-vehicle control (249.36 ± 10.56 fmol/well). Treatment of C-20/A4 chondrocytes with the pan melanocortin receptor agonist α-MSH (1.0 – 30.0 μg/ml), for 30 mins caused a significant increase in intracellular cAMP at 3.0, 10.0 and 30.0 μg/ml by approximately 2-fold, 2.6-fold and 1.7-fold increase to 568.86 ± 22.74 fmol/well ($p \leq 0.001$), 638.6 ± 41.6 fmol/well ($p \leq 0.001$) and 429.1 ± 18.9 fmol/well ($p \leq 0.05$) respectively compared to the vehicle control level of 249.36 ± 10.56 fmol/well (Figure 3.23 A)

Similarly, a drastic 3.2-fold increase in cAMP accumulation peaking at 800.83 ± 30.0 fmol/well ($p \leq 0.001$) was recorded following treatment with 3.0 μg/ml of the highly selective MC_3 receptor agonist D[TRP]^8-γ-MSH (Figure 3.23 B). A significant increase of 3-fold compared to vehicle control levels was observed at 10.0 μg/ml (789.9 ± 49.15 fmol/well, $p \leq 0.001$) and 30.0 μg/ml (696.2 ± 27.59 fmol/well, $p \leq 0.001$). In comparison, the $MC_{3/4}$ receptor antagonist SHU9119 (1.0 – 10.0 μg/ml) failed to induce a statistically significant cAMP response in C-20/A4 cells at any of the concentrations tested (Figure 3.23 C).

The MC_5 full agonists PG901 and PG911 (1.0 –10.0 μg/ml) were then evaluated to determine whether MC_5 was a functionally active receptor. Both are full agonists at the MC_5 (EC(50) = 0.072 nM and 0.031 nM, respectively), but full antagonists at the $MC_3/_4$. However, no statistically significant cAMP formation was recorded following stimulation at any of the concentrations (Figure 3.23 D, E).

Following identification that α-MSH and D[TRP]^8-γ-MSH induced cAMP accumulation in C-20/A4 chondrocytes, the peptides were evaluated in the presence of the MC$_{3/4}$ antagonist SHU9119 to determine if the cAMP accumulation occurred *via* these receptors (Figure 3.24). C-20/A4 chondrocytes were incubated with α-MSH and D[TRP]^8-γ-MSH (0 – 30.0 μg/ml) alone or in the presence of SHU9119 (10.0 μg/ml).

SHU9119 totally abrogated the effect elicited by D[TRP]^8-γ-MSH, whilst cAMP formation triggered by α-MSH was only slightly reduced at 3.0 µg/ml (Figure 3.24 B). SHU9119 inhibited the activation of the MC$_3$ elicited by D[TRP]^8-γ-MSH at all concentrations evaluated; where at 3.0 µg/ml of the antagonist, only slight inhibition resulted leading it to drop from 379.82 ± 15.29 fmol/well to 320.21 ± 30.35 fmol/well (n/s, *p*> 0.05). The addition of the antagonist with 3.0 µg/ml D[TRP]^8-γ-MSH caused the cAMP levels to fall from 800.83 ± 29.99 to 318.84 ± 9.34 fmol/well, representing ~ 87 % reduction (*p*≤ 0.001).

Similarly, SHU9119 (10.0 µg/ml) inhibition led to a >84 % reduction in cAMP accumulation elicited by 10.0 µg/ml D[TRP]^8-γ-MSH (*p*≤ 0.01) with a reduction from 789.9 ± 49.2 fmol/well down to 338.04 ± 10.6 fmol/well cAMP accumulation for D[TRP]^8-γ-MSH and D[TRP]^8-γ-MSH + SHU9119 (10.0 µg/ml). Even the highest concentration of the selective MC$_3$ agonist, had no effect when given simultaneously with the MC$_{3/4}$ receptor antagonist: the concentration of cAMP was reduced from 696.21 ± 27.59 to 290.12, illustrating an 80 % reduction in cAMP accumulation (*p*≤ 0.001). cAMP levels triggered by α-MSH, were not reduced by SHU9119, except when the antagonist was added in conjunction with 3.0 μg/ml of α-MSH, when SHU9119 (10.0 μg/ml) led to 15.5% decrease in cAMP formation from 568.86 ± 22.74 to 480.29 ± 35.3 (n.s., *p*> 0.05; Figure 3.24 A).
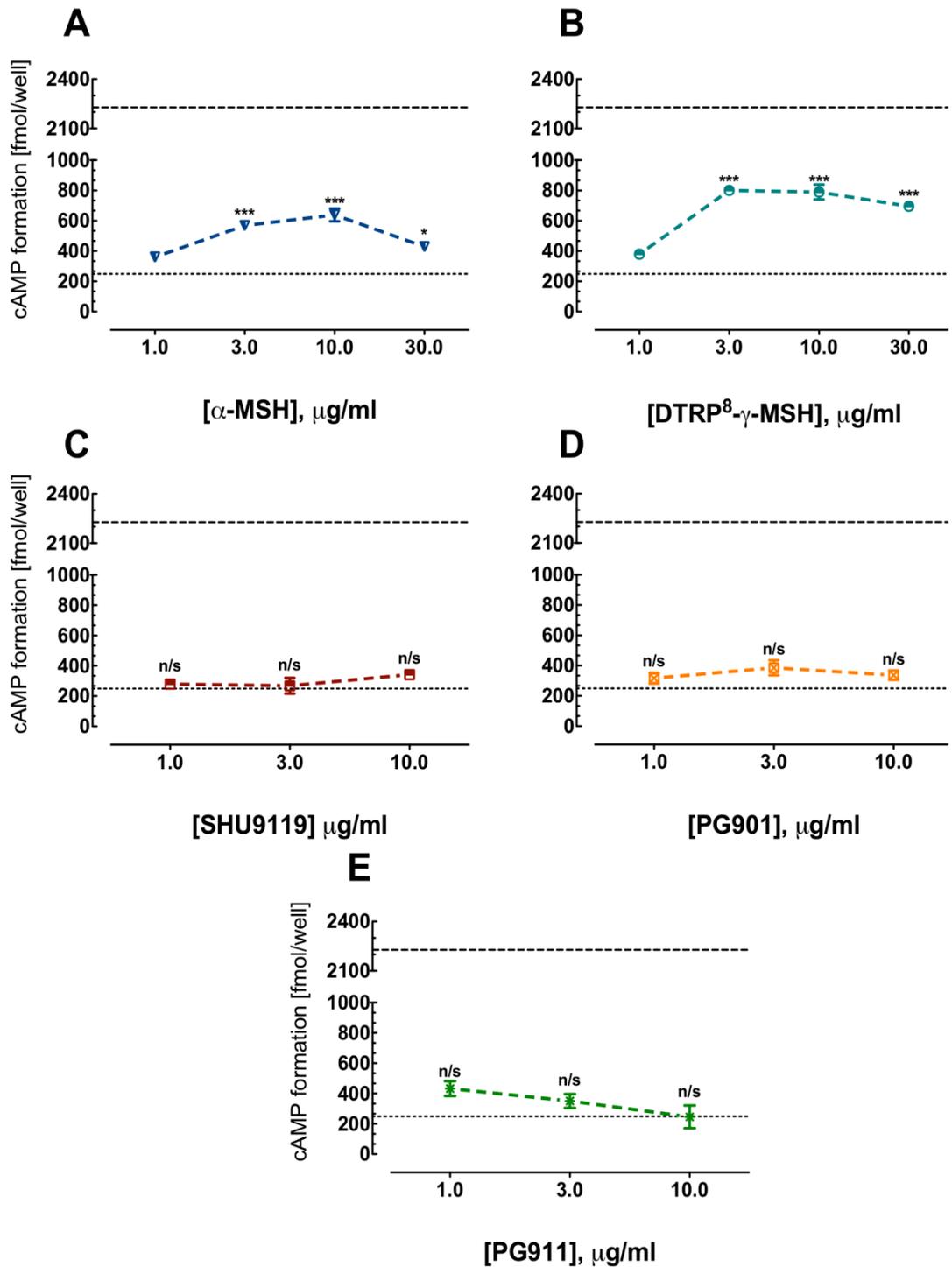
Figure 3.23. MC$_1$, MC$_3$, MC$_5$ functionality determined by cAMP EIA.

C-20/A4 chondrocytes were treated with 1.0-30.0 μg/ml α-MSH **(A)**, [DTRP^8]-γ-MSH **(B)** and with 1.0-10.0 μg/ml SHU9119 **(C)**, PG-901 **(D)** and PG911 **(E)** for 30 min and cAMP (fmoles/well) accumulation determined by cAMP EIA. Dotted lines indicate basal accumulation in PBS-treated whilst dashed lines indicate maximal accumulation in 3.0 μM FSK-treated C20/A4 cells. Data is Mean ± SEM of n=6 samples, n.s. $p > 0.05$, *$p ≤$ 0.05, ***$p ≤$ 0.001) vs. PBS-treated control cultures.
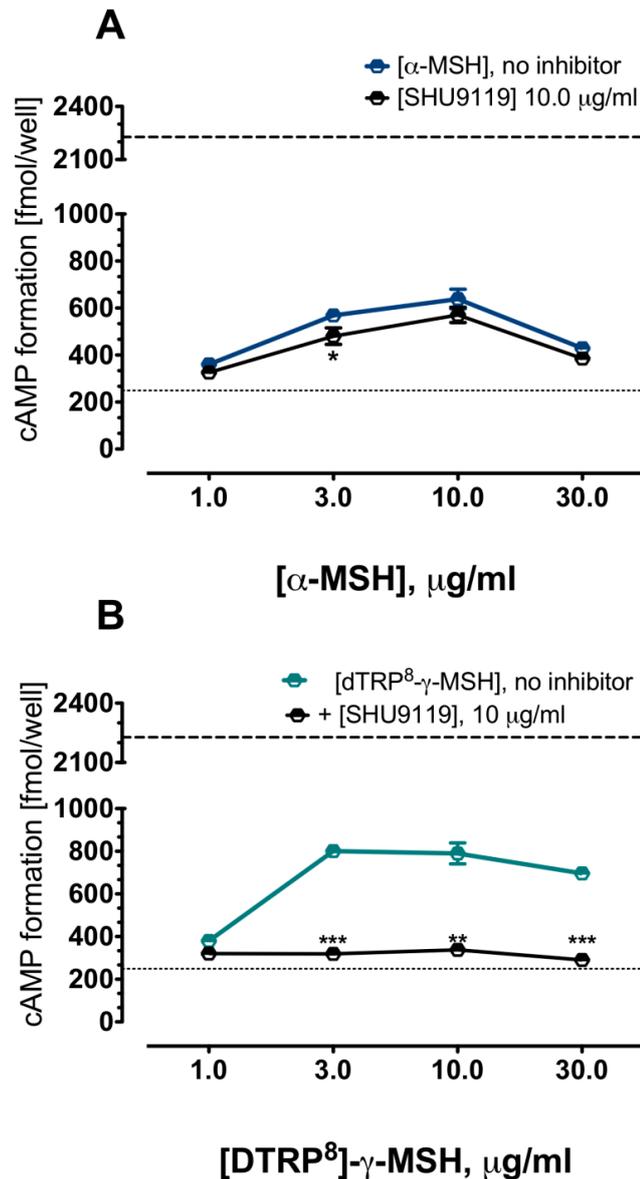
Figure 3.24. SHU9119 prevents D[TRP]^8-γ-MSH from activating MC$_3$, but has no effect on MC$_1$ functionality.

C-20/A4 chondrocytes were treated with 1.0-30.0 μg/ml α-MSH (Panel A) and [DTRP^8]-γ-MSH (Panel B) alone or in the presence of SHU9119 (10 μg/ml) for 30 min and cAMP (fmoles/well) accumulation determined by cAMP EIA. Dotted lines indicate basal accumulation in PBS-treated, whilst dashed lines indicate maximal accumulation in FSK-treated C20/A4 cells. Data is Mean ± SEM of n=6 samples, *p≤ 0.05, ***p≤ 0.01 vs. PBS-treated control cultures.

3.3.3 Modulation of pro-inflammatory mediator release and apoptosis by melanocortin peptides on TNF-α stimulated C20/A4 chondrocytes.

3.3.3.1 Effect of melanocortin peptides on basal release of pro-inflammatory cytokines production from C-20/A4 chondrocytes.

Following identification of functionally active MC_1 and MC_3 on C-20/A4 chondrocytes, their effect on basal cytokine production was determined. C-20/A4 chondrocytes were treated with α-MSH (3.0 μg/ml), D[TRP]^8-γ-MSH (3.0 μg/ml) and SHU9119 (10.0 μg/ml), and the basal levels of production of the pro-inflammatory cytokines IL-1β, IL-6, IL-8 and MCP-1 were determined by cytokine ELISAs (Table 3.3). Treatment of cells with melanocortin peptides did not cause significant alteration compared to control cultures treated with DMEM alone ($p>$ 0.05).

Table 3.3 Effect of melanocortin derived peptides on basal levels of pro-inflammatory cytokine release from C-20/A4 chondrocytes.

	IL-1β	IL-6	IL-8	MCP-1
DMEM Media	2.77± 0.44	21.09±18.5	72.65±4.78	46.18 ± 14.12
α-MSH (3.0 μg/ml)	5.76±1.8	38.29±29.1	82.3±5.36	54.26 ± 11.11
D[TRP]^8-γ-MSH (3.0 μg/ml)	3.69±0.65	23.19±11.65	67.76±6.3	48.42± 7.1
SHU9119 (10.0 μg/ml)	4.55±0.55	37.19±2.97	85.58±4.85	54.41±11.93

Data are Means ± SEM of n=4 of three determinations.

Interleukin(IL)-1β; IL-6, Interleukin(IL)-6, interleukin(IL)-8; MCP-1, monocyte chemoatractant protein-1

3.3.3.2 Attenuation of TNF-α-activated pro-inflammatory cytokines release from C-20/A4 chondrocytes by α-MSH and D[TRP]^8-γ-MSH.

The identification of the melanocortin receptors' functionality on C-20/A4 chondrocytes and their non-cytotoxic effects, led to the evaluation of α-MSH and D[TRP]^8-γ-MSH effect on pro-inflammatory cytokine release following TNF-α-stimulation.

The C-20/A4 cell-line was treated with α-MSH or D[TRP]^8-γ-MSH in concentrations ranging from 0.3 – 30.0 μg/ml for 30 mins prior to stimulation with TNF-α, cell free supernatants were collected at 2, 6 and 24 h post-stimulation and pro-inflammatory cytokines IL-1β, IL-6 and IL-8 were determined by commercially available ELISAs.

α-MSH led to a significant concentration-dependent decrease in IL-1β concentration with a maximal reduction detected at 3.0 μg/ml α-MSH with 88.6 ± 3.2 % at 2 h ($p \leq$ 0.001) and 84.8 ± 4.01 % at 6 h ($p \leq$ 0.001) compared to TNF-α-stimulated controls. The melanocortin peptide did not modulate IL-1β release at 24 h post-stimulation (Figure 3.25 A). The MC$_3$ agonist D[TRP]^8-γ-MSH (3.0 μg/ml) led to the maximal observed reduction of 72.73 ± 3.31 % at 6 h (Figure 3.25 B).

Evaluating the effects of α-MSH on IL-6 showed that after 6 h, α-MSH caused a concentration-dependant effect peaking at 3.0 μg/ml with 72.1 ± 2.3 % compared to TNF-α-stimulated controls. It was observed that higher concentrations did not elicit a significant effect. α-MSH did not exert any detectable effect at 24 h post-stimulation, except when C-20/A4 chondrocytes were treated with low concentration of 0.3 μg/ml with 37 ± 3.4 % and 34.5 ± 4.6 % inhibition of IL-6 respectively ($p \leq$ 0.05). D[TRP]^8-γ-MSH caused a concentration-dependent response observed at all time-points (Figure 3.26 B). The maximum effect at 2 h was observed following stimulation with 0.3 and 3.0 μg/ml with 75.0 ± 6.9 % and 73.1 ± 3.5 % reduction respectively. At 6 h, 3.0 μg/ml were identified as the most potent concentrations leading to 61.3 ± 2.1 % and 60.2 ± 2.6 % inhibition of IL-6

synthesis respectively. The peptide was still effective at 24 h post stimulation with a pronounced concentration-dependent reduction of IL-6 concentration, peaking with 85 ± 2.0 % inhibition at 30.0 μg/ml (Figure 3.26 B).

α-MSH inhibited IL-8 release in a concentration dependent manner at 2 and 6 h and was not effective at later time points (Figure 3.27 A). At 2 h, the greatest reduction in IL-8 synthesis was caused by 3.0, and 30.0 μg/ml of α-MSH with 59.2 ± 5.5 % and 66.7 ± 9.85 % decrease respectively, while 0.3 had less effect leading to 47.2 ± 9.6 % down-regulation. There was a concentration dependent inhibition of IL-8, 6 h post stimulation, with a maximal reduction of 60.21 ± 2.1% caused by 3.0 μg/ml α-MSH.

D[TRP]^8-γ-MSH (0.3 μg/ml) failed to suppress the effect of TNF-α, except at 24 h post-stimulation, when it caused 51% decrease in the cytokine production (Figure 3.27 B). The higher concentration of 3.0 μg/ml, led to significant down-regulation of IL-8 release regardless of the time point evaluated with a 67.32 ± 2.76%, 44.71 ± 5.14 % and 37.11 ± 2.4 % reductions at 2, 6 and 24h respectively. Following treatment of C-20/A4 chondrocyte with 30.0 μg/ml of D[TRP]^8-γ-MSH a consistent decrease of 80.72 ± 2.13 % ($p \leq 0.01$), 64.72 ± 2.3 % ($p \leq 0.001$) and 69.89 ± 3.23 % ($p \leq 0.01$) was observed at 2, 6 and 24 h post-stimulation respectively.
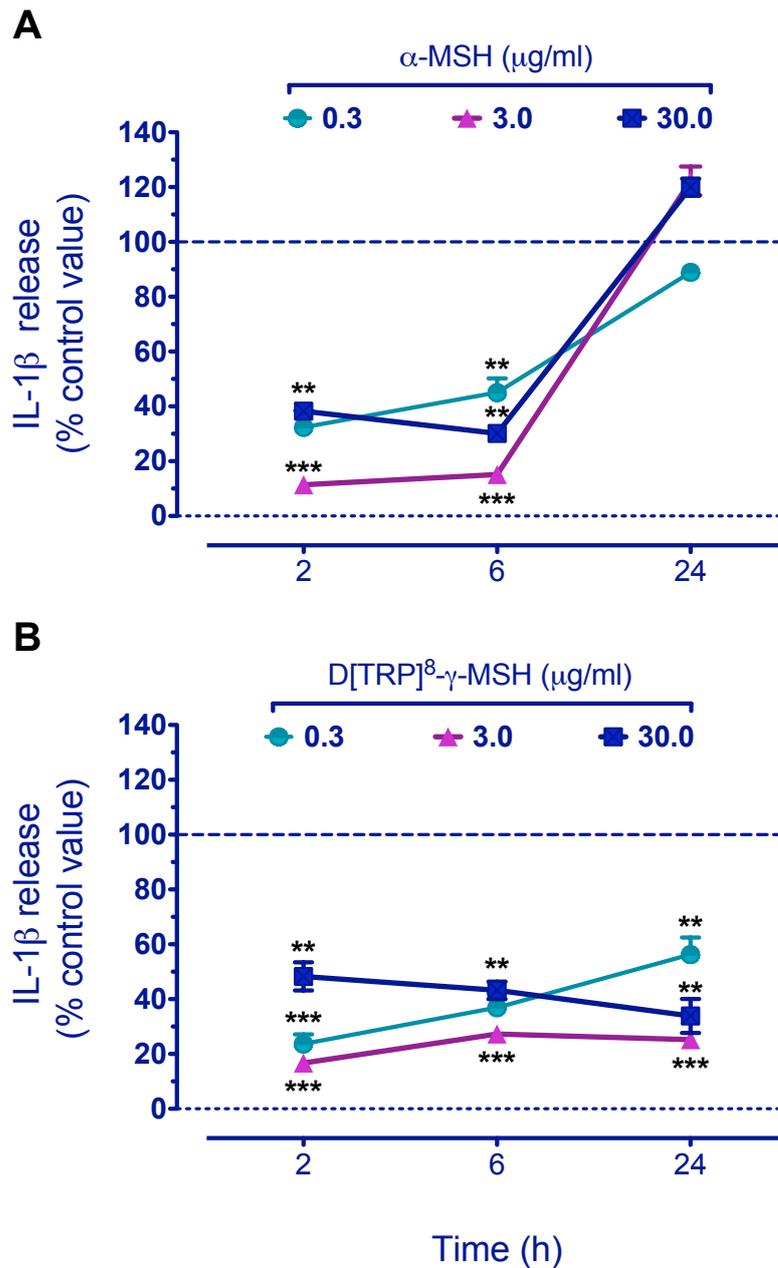
Figure 3.25. Effect of α-MSH and D[TRP]^8-γ-MSH on TNF-α induced IL-1β release from C-20/A4 cells.

C-20/A4 chondrocytes were pre-treated for 30 min with PBS (Dotted line), α-MSH or D[TRP]^8-γ-MSH (0.3 – 30.0 μg/ml) prior to stimulation with TNF-α (60.0 pg/ml; dashed line) and cell-free supernatants collected at 2, 6 and 24 h post stimulation and analysed for IL-1β by ELISA. Data are presented as Mean ± SEM of n=4 independent experiments repeated in triplicate, *p≤ 0.05, **p≤ 0.01, ***p≤ 0.001.
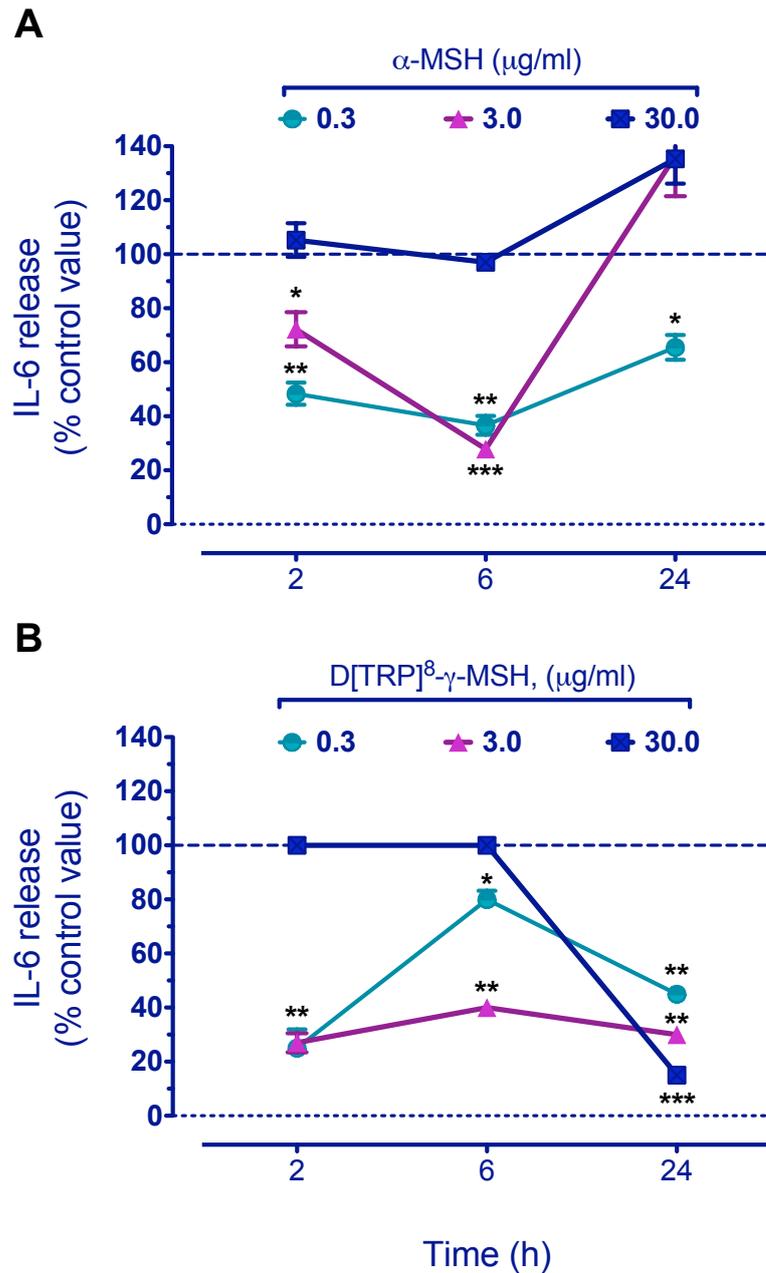
Figure 3.26. Effect of α-MSH and D[TRP]^8-γ-MSH on TNF-α induced IL-6 release from C-20/A4 cell-line.

C-20/A4 chondrocytes were pre-treated for 30 min with PBS (Dotted line), α-MSH or D[TRP]^8-γ-MSH (0.3 – 30.0 μg/ml) prior to stimulation with TNF-α (60.0 pg/ml; dashed line) and cell-free supernatants collected at 2, 6 and 24 h post stimulation and analysed for IL-6 by ELISA. Data are presented as Mean ± SEM of n=4 independent experiments repeated in triplicate, *p≤ 0.05, **p≤ 0.01, ***p≤0.001.
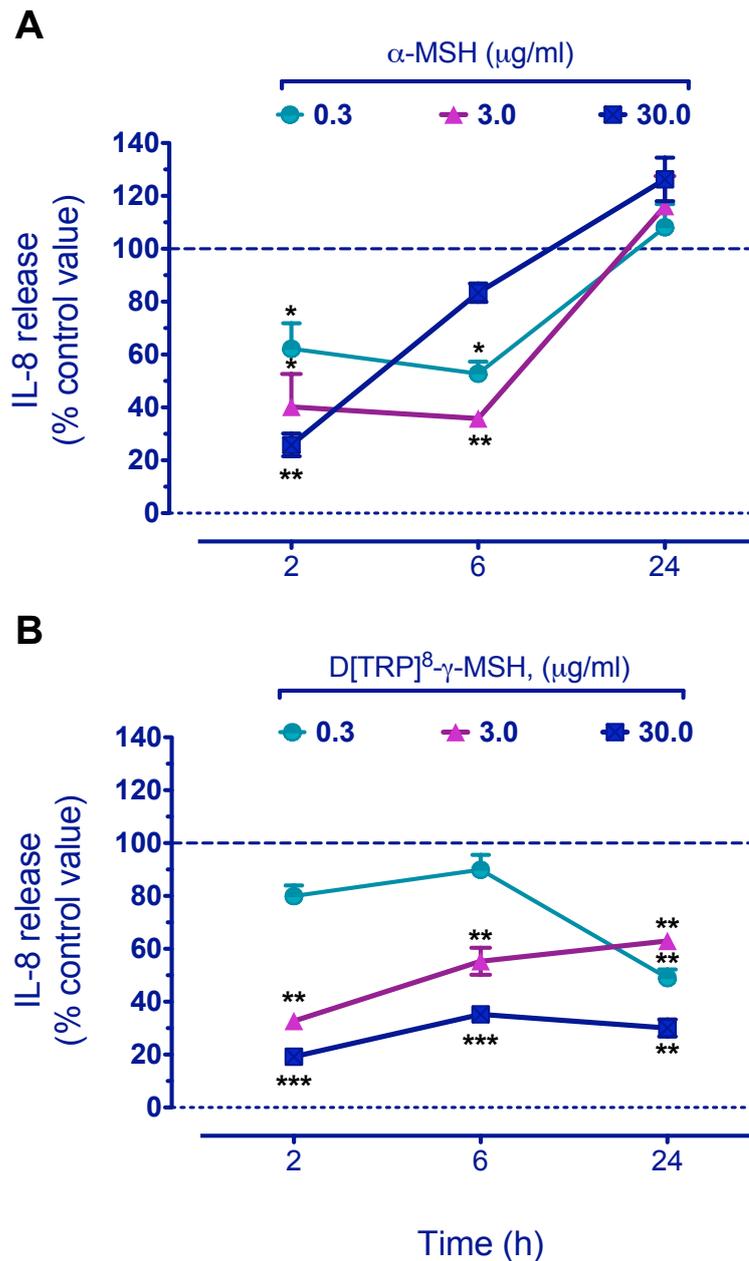
Figure 3.27. Effect of α-MSH and D[TRP]^8-γ-MSH on TNF-α induced IL-8 release from C-20/A4 cell-line.

C-20/A4 chondrocytes were pre-treated for 30 min with PBS, α-MSH (Panel A) and D[TRP]^8-γ-MSH (0.3 – 30.0 μg/ml, Panel B) prior to stimulation with TNF-α (60.0 pg/ml) and cell-free supernatants collected at 0, 2, 6 and 24 h post stimulation and analysed for IL-8 by ELISA. Data are presented as Mean ± SEM of n=4 independent experiments repeated in triplicate, *$p \leq$ 0.05, **$p \leq$ 0.01, ***$p \leq$ 0.001.

3.3.3.3 Effect of PG901 and PG911 on TNF-α stimulated pro-inflammatory cytokines release from C-20/A4 chondrocytes.

Following the finding that MC$_5$ was not functionally active, the effects of the selective agonist PG901 and PG911 on cytokine release was evaluated to ensure that the anti-cytokine effects elicited by α-MSH were independent of MC$_5$. C-20/A4 chondrocytes were pre-treated with PG901 or PG911 (3.0 or 10.0 μg/ml) prior to stimulation with TNF-α (60.0 pg/ml) and were incubated for 6 h. Subsequently, IL-1β, IL-6, IL-8 and MCP-1 release was determined by ELISA (Table 3.1). As previously observed (Figures 3.5, 3.6 and 3.8), TNF-α (60.0 pg/ml) caused a significant release of these cytokines compared to control cells ([†]$p \leq 0.05$), IL-1β, IL-6 and MCP-1 were not inhibited by PG901 or PG911 at any concentration evaluated. However, a different scenario was observed with respect to IL-8 only PG911 (10.0 μg/ml) caused a 40.4 ± 23.3 % reduction; all other concentrations of PG911 and PG901 were inactive.

Table 3.1 Effect of PG901 and PG911 on TNF-α induced cytokine release from C-20/A4 cell-line.

	IL-1β	IL-6	IL-8	MCP-1
Medium	2.77± 0.44	21.09±18.5	72.65±4.78	46.18 ± 14.12
TNF-α (60 pg/ml)	24.37±8.69[†]	280.6 ± 20.39[†]	202.82 ± 21.04[†]	101.2± 16.37[†]
TNF-α + PG901 (3.0 μg/ml)	17.56 ± 1.03	231.29 ± 24.19	165.41 ± 23.81	84.6 ± 16.37
TNF-α + PG901 (10.0 μg/ml)	19.43 ± 3.29	253.62 ± 26.46	144.84 ± 25.41	89.96 ± 18.91
TNF-α + PG911 (3.0 μg/ml)	19.92 ±4.99	273.396 ± 48.21	181.37 ± 54.78	97.05 ± 5.29
TNF-α + PG911 (10.0 μg/ml)	16.38 ± 2.01	244.29 ± 17.14	**122.46 ± 18.47***	85.88 ± 6.05

Data are Means ± SEM of n=4 of three determinations [†,] *$p \leq 0.05$.

IL-1β, Interleukin 1β; IL-6, inteleukin-6; IL-8, interleukin-8; MCP-1, monocyte chemoatractant protein-1.

3.3.4 Effect of SHU9119 on α-MSH and D[TRP]^8-γ-MSH modulation of pro-inflammatory cytokines from C-20/A4 chondrocytes.

The effect of the MC$_{3/4}$ antagonist SHU9119 on α-MSH and D[TRP]^8-γ-MSH modulation of cytokine release was evaluated to determine whether MC$_1$ or MC$_3$ were transmitting the anti-inflammatory effects of these peptides in the chondrocyte system. C-20/A4 chondrocytes were pre-treated with α-MSH or D[TRP]^8-γ-MSH (3.0 μg/ml) $\pm$ SHU9119 (10.0 μg/ml) for 30 mins prior to stimulation with TNF-α (60.0 pg/ml) and total RNA and cell-free supernatants were collected at 6 h post-stimulation. Total RNA was extracted from the treated C-20/A4 chondrocytes and PCR amplification performed using *IL6* and *IL8* specific primers (Table 2.9).

3.3.4.1 Antagonistic effect of SHU9119 on *IL6* and *IL8* gene expression following α-MSH and D[TRP]^8-γ-MSH stimulation

Densitometric quantification (Figure 3.28 A) showed that α-MSH (3.0 μg/ml) significantly down-regulated TNF-α-induced *IL6* and *IL8* expression, therefore causing a 35 % drop in *IL6* expression from 0.802 $\pm$ 0.025 to 0.517 $\pm$ 0.018 au ($p \leq$ 0.05) and a 24.5 % down-regulation of *IL-8* from 1.92 $\pm$ 0.1 au down to 1.45 $\pm$ 0.028 au ($p \leq$ 0.05), this effect was not blocked by SHU9119 (Figure 3.28 B and C).

The MC$_3$ agonist D[TRP]^8-γ-MSH significantly inhibited the transcription of both *IL6* and *IL8* genes as shown by densitometric quantification (Figure 3.29 A) with a 27 % inhibition of *IL6* expression from 0.802 $\pm$ 0.025 au to 0.585 $\pm$ 0.02 au ($p \leq$ 0.05) and 35.7 % reduction in *IL8* transcription from 1.92 $\pm$ 0.1 to 1.234 $\pm$ 0.016 au ($p \leq$ 0.01). SHU9119 completely inhibited the effect of D[TRP]^8-γ-MSH on *IL6* and *IL8*, such that *IL6* expression was 0.94 $\pm$ 0.05 au and *IL-8* was 1.85 $\pm$ 0.19 au ($p \leq$ 0.05; Figure 3.29 B and C).

3.3.4.2 Effect of SHU9119 on cytokine release from α-MSH and D[TRP]^8-γ-MSH pre-treated TNF-α-activated C-20/A4 chondrocytes.

In order to further elucidate the function of the melanocortin peptides α-MSH and D[TRP]^8-γ-MSH on TNF-α stimulated cytokine production, the MC$_{3/4}$ antagonist SHU9119 was used to selectively block the function of MC$_3$. C-20A4 cells were pre-treated with 3.0 μg/ml of α-MSH or D[TRP]^8-γ-MSH for 30 min prior to stimulation with TNF-α (60.0 pg/ml) for 6 h and IL-6, IL-8 and MCP-1 levels were detected by ELISA. α-MSH (3.0 μg/ml) caused a marked reduction in IL-6, causing a 72.13 $\pm$ 2.3 % ($p \leq$ 0.01; Figure 3.30 A), 60.22% ($p \leq$ 0.01) reduction in IL-8 production (Figure 3.30 B) and a 21.3 % reduction in MCP-1 ($p \leq$ 0.05; Figure 3.30C). These effects were not abrogated in the presence of SHU9119 (Figure 3.30 A-C).

The selective MC$_3$ agonist D[TRP]^8-γ-MSH inhibited IL-6 production by 68 % ($p \leq$ 0.01), 44.72 % ($p \leq$ 0.01) reduction in IL-8 secretion and a 26 % ($p \leq$ 0.01) reduction in MCP-1 ($p \leq$ 0.05) an effect blocked by SHU9119 (Figure 3.30 D-F).
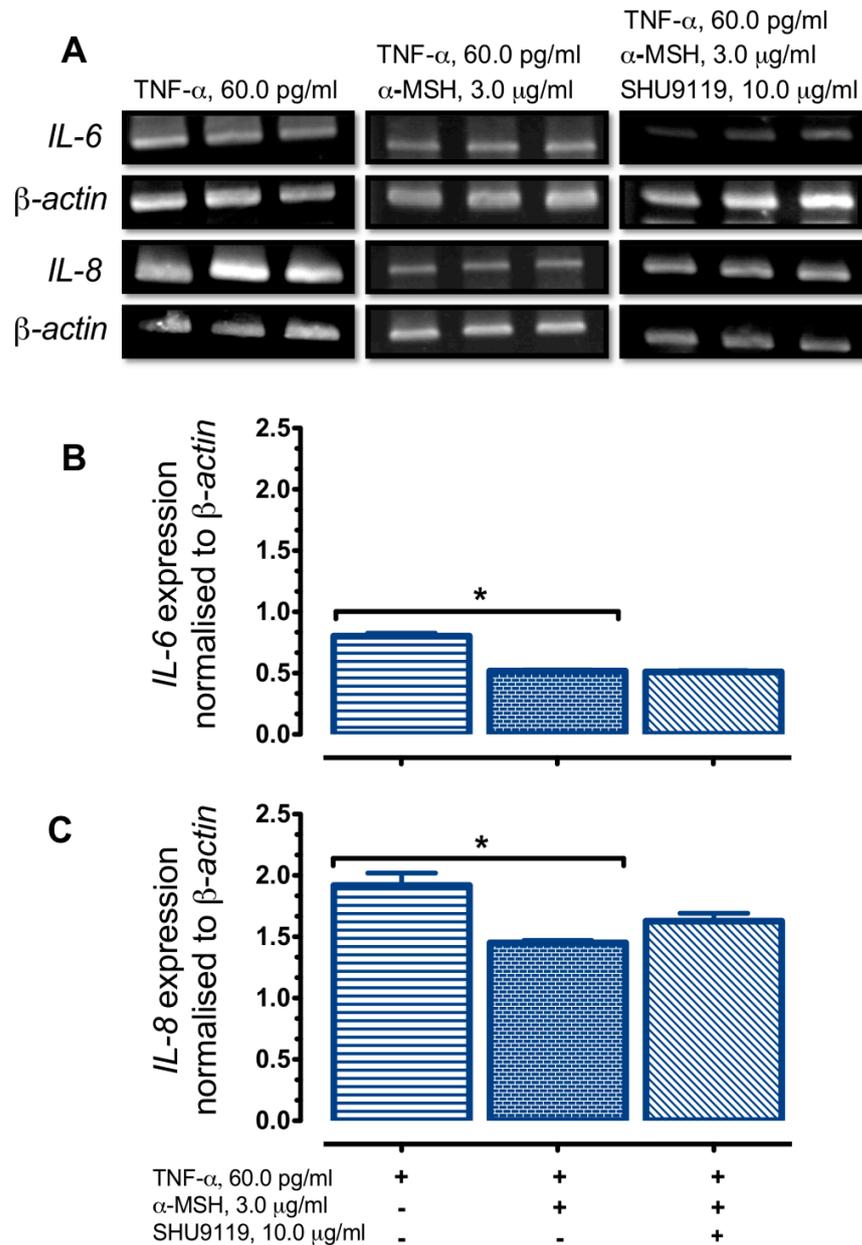
Figure 3.28. Densitometric quantification of *IL-6* and *IL-8* mRNA expression in TNF-α-activated C-20/A4 chondrocytes treated with α-MSH and SHU9119 for 6 h.

C-20/A4 chondrocytes were pre-treated for 30 min with α-MSH (3.0 μg/ml) ± SHU9119 (10.0 μg/ml) prior to stimulation with TNF-α (60.0 pg/ml) and total RNA extracted 6 h post stimulation. PCR amplification with the respective primers for *IL-6* and *IL-8* was used to detect and quantify gene expression on 2 % agarose gels in triplicates with *β-actin* used as internal control (Panel A). Comparison of densitometrically quantified *IL-6* and *IL-8* expression following treatments (Panel B) shown in arbitrary units, each value normalized to the respective *β-actin* expression. Data are presented as Mean ± SEM of n=4 independent experiments *$p \leq$ 0.05.

A

TNF-α, 60 pg/ml

TNF-α, 60 pg/ml
[DTRP[8]]-γ-MSH,
3.0 μg/ml

TNF-α, 60 pg/ml
[DTRP[8]]-γ-MSH
SHU9119, 10.0 μg/ml

IL-6

β-*actin*

IL-8

β-*actin*

B

IL-6 expression normalised to β-actin

C

IL-8 expression normalised to β-actin

TNF-α, 60.0 pg/ml	+	+	+
dTRP[8]–γ–MSH, 3.0 μg/ml	-	+	+
SHU9119, 10.0 μg/ml	-	-	+

Figure 3.29. Densitometric quantification of *IL-6* and *IL-8* mRNA expression in TNF-α-activated C-20/A4 chondrocytes treated with D[TRP][8]-γ-MSH and SHU9119 for 6 h.

C-20/A4 chondrocytes were pre-treated for 30 min with D[TRP][8]-γ-MSH (3.0 μg/ml) ± SHU9119 (10.0 μg/ml) prior to stimulation with TNF-α (60.0 pg/ml) and total RNA extracted 6 h post stimulation. PCR amplification with the respective primers for IL-6 and IL-8 was used to detect and quantify gene expression on 2 % agarose gels in triplicates with β-*actin* used as internal control (Panel A). Comparison of densitometrically quantified *IL-6* and *IL-8* expression following treatments (Panel B) shown in arbitrary units, each value normalized to the respective β-*actin* expression. Data are presented as Mean ± SEM of n=4 independent experiments *$p \leq 0.05$, **$p \leq 0.01$).

Figure 3.30. Effect of SHU9119 on IL-6, IL-8 and MCP-1 release from α-MSH and D[TRP]⁸-γ-MSH pre-treated C-20/A4 chondrocytes.

C-20/A4 chondrocytes were pre-treated for 30 min with PBS, α-MSH (Panel A – C) or D[TRP]⁸-γ-MSH (3.0 µg/ml) alone (Panels D – F) or in the presence of SHU9119 (10.0 µg/ml) prior to stimulation with TNF-α (60.0 pg/ml) and cell-free supernatants collected at 6 h post stimulation and analysed for IL-6, IL-8 and MCP-1 by ELISA. Data are presented as Mean ± SEM of n=4 independent experiments repeated in triplicate, *p≤ 0.05, **p≤ 0.01.

3.3.4.3 Effect of SHU9119 on *MMP1, MMP3* and *MMP13* expression in α-MSH D[TRP]^8-γ-MSH and pre-treated TNF-α activated chondrocytes.

Following the identification of the anti-cytokines effects of α-MSH and D[TRP]^8-γ-MSH, their effect was evaluated on matrix metalloproteinases expression (Figure 3.31A). α-MSH (3.0 μg/ml) caused a significant ~80 % down-regulation in the transcription of *MMP1;* densitometric quantification of the bands revealed that expression was reduced from 1.71 ± 0.11 au to 0.34 ± 0.04 au ($p \leq$ 0.001; Figure 3.31 B). *MMP3* expression was reduced by α-MSH by 48 % from 0.37 ± 0.02 au (TNF-α-treated control cultures) to 0.19 ± 0.03 au ($p \leq$ 0.01; Figure 3.31C), whilst *MMP13* expression was inhibited by 66.7 % from 0.24 ± 0.01 au (TNF-α, 60.0 pg/ml) to 0.083 ± 0.002 au (Figure 3.31 D).

This inhibition of *MMP1, MMP3* and *MMP13* was not modified in the presence of SHU9119. Nevertheless, when SHU9119 (10.0 μg/ml) was added together with α-MSH (3.0 μg/ml), there was a marked synergistic down-regulation of *MMP13* synthesis, with the combination causing a marked inhibition of 87.5 % ($p \leq$ 0.001) compared to TNF-α treated controls and 57.8 % drop compared to samples pre-treated with α-MSH alone ($p \leq$ 0.05).

Treatment of C-20/A4 chondrocytes with D[TRP]^8-γ-MSH (3.0 μg/ml) led to a significant 88.9 % reduction in *MMP1* expression (0.189 ±0.1 au; $p \leq$ 0.001; Figure 3.32A) an effect blocked completely by co-administration of SHU9119 (10.0 μg/ml; Figure 3.32 B). *MMP3* was reduced by 76 % (0.098 ± 0.006 au; $p \leq$ 0.01) compared to TNF-α stimulated cultures (0.372 ± 0.012 au) an effect blocked by SHU9119 (10.0 μg/ml), returning the expression levels back to TNF-α-stimulated levels (0.372 ± 0.012 au; Figure 3.32 C). A similar observation was noted for *MMP13* gene expression, where D[TRP]^8-γ-MSH (3.0 μg/ml) caused a 91.7 % drop in transcription levels (0.02 ± 0.004 au; $p \leq$ 0.001) as compared to TNF-α–treated controls (0.237 ± 0.008 au), an effect completely abolished by SHU9119 (10.0 μg/ml; Figure 3.32 D) .
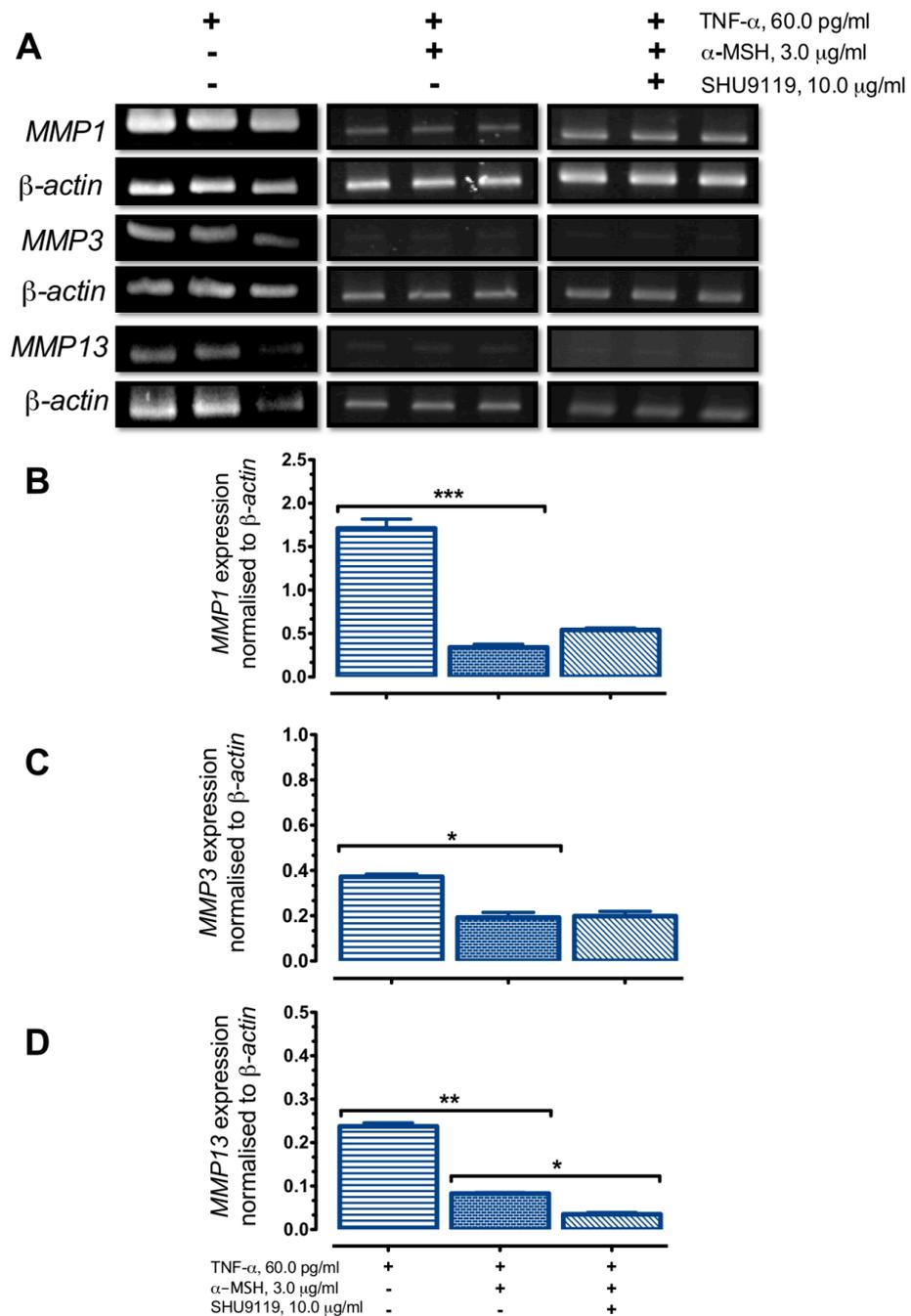
Figure 3.31. Densitometric quantification of MMPs mRNA expression in C-20/A4 cells treated with α-MSH and SHU9119 for 6 h.

C-20/A4 chondrocytes were pre-treated for 30 min with α-MSH (3.0 μg/ml) ± SHU9119 (10.0 μg/ml) and stimulated with TNF-α (60.0 pg/ml). Total RNA was extracted at 6 h post stimulation and PCR amplification with the respective primers for *MMP1, MMP3* and *MMP13* was used to detect and quantify gene expression on 2 % agarose gels in triplicates, with *β-actin* used as internal control (Panel A). Comparison of densitometrically quantified *MMP1, MMP3* and *MMP13* expression for α-MSH ± SHU9119 (Panel B, C and D) shown in arbitrary units, each value normalized to the respective *β-actin* expression. Data is presented as Mean ± SEM of n=4 independent experiments *$p \leq$ 0.05, **$p \leq$ 0.01, ***$p \leq$ 0.001).

Figure 3.32. Densitometric quantification of MMPs mRNA expression in C-20/A4 cells treated with D[TRP]^8-γ-MSH and SHU9119 for 6 h.

C-20/A4 chondrocytes were pre-treated for 30 min with D[TRP]^8-γ-MSH (3.0 μg/ml) ± SHU9119 (10.0 μg/ml) and stimulated with TNF-α (60.0 pg/ml). Total RNA was extracted at 6 h post stimulation and PCR amplification with the respective primers for *MMP1, MMP3* and *MMP13* was used to detect and quantify gene expression on 2 % agarose gels in triplicates with *β-actin* used as internal control (Panel A). Comparison of densitometrically quantified *MMP1, MMP3* and *MMP13* expression for [DTRP]^8-γ-MSH ± SHU9119 (Panel B, C and D) shown in arbitrary units, each value normalized to the respective *β-actin* expression. Data is presented as Mean ± SEM of n=4 independent experiments *$p≤0.05$, **$p≤0.01$, ***$p≤0.001$).

174

3.3.5 Evaluation of the effect of melanocortin peptides on anti-inflammatory protein synthesis and release from C-20/A4 chondrocytes.

Human chondrocytes from healthy and osteoarthritic cartilage have been previously shown to express both IL-10 as well as the IL-10 receptor (IL10R; Iannone *et al.*, 2001). IL-10 has potent anti-inflammatory properties, repressing the expression of inflammatory cytokines such as TNF-α, IL-6 and IL-1β by activated cells. Direct stimulation of OA chondrocytes with IL-10 has been shown to inhibit the activation of chondrocytes by TNF-α and therefore to down-regulate the expression of *MMP1* and *MMP13* (Shlopov *et al.*, 2000). In addition, IL-10 has been shown to induce HO-1, which is implicated in the protection against tissue damage and is repressing pro-inflammatory cytokines such as TNF-α. Therefore, following identification of the anti-inflammatory properties of α-MSH and D[TRP]^8-γ-MSH on TNF-α stimulated C20/A4 chondrocytes, their effect on the production of these anti-inflammatory proteins was next evaluated.

3.3.5.1 Effect of melanocortin peptides alone on IL-10 release from C-20/A4 chondrocytes.

IL-10 protein levels in cell-free supernatants were determined by ELISA following stimulation of C-20/A4 chondrocytes with a panel of melanocortin peptides (α-MSH, D[TRP]^8-γ-MSH, PG901, PG911 all at 3.0 μg/ml and SHU9119 (10.0 μg/ml) for 0, 2, 6 and 24 h.

Figure 3.33 A and B demonstrates the time-dependent increase in IL-10 release peaking at 6 h following α-MSH (3.0 μg/ml) and D[TRP]^8-γ-MSH (3.0 μg/ml) treatment. At time 0 there was almost no basal release of IL-10 (1.625 ± 0.92 pg/ml). However, following stimulation with α-MSH and D[TRP]^8-γ-MSH for 2 h, there was a significant 15.5-fold (25.28 ± 2.25 pg/ml, *$p \leq$ 0.05) and 12-fold (19.96 ± 3.25 pg/ml; *$p \leq$ 0.05) increase in IL-10 production for both peptides respectively. IL-10 levels steadily increased to 37.9 ± 4.13 pg/ml and 25.92 ± 2.3 pg/ml following stimulation with α-MSH and D[TRP]^8-γ-MSH for 6 h respectively. At 24 h post-treatment, α-MSH and D[TRP]^8-γ-MSH treatment caused IL-10 concentrations to return to levels similar to those observed at 2h (15.67 ± 3.93

pg/ml and 19.23 ± 4.76 pg/ml, *$p \leq$ 0.05 both peptides). At all time points SHU9119, PG901 and PG911 (10.0 µg/ml) failed to induce any significant increase in IL-10 above basal production at time 0h (Figure 3.33 C-E).

3.3.5.2 Effect of melanocortin peptides on IL-10 production from TNF-α-activated C-20/A4 chondrocytes.

Pre-treatment of TNF-α–activated C-20/A4 chondrocytes with α-MSH (0.3 – 30.0 µg/ml) for 2h caused a concentration-dependent increase in IL-10, peaking with 37.5 ± 2.83 pg/ml following addition of 3.0 µg/ml α-MSH. Concentrations higher than 3.0 µg/ml didn't cause significant increases in IL-10 compared to untreated controls (1.625 ± 0.919 pg/ml). At 6 h post stimulation α-MSH caused a significant increase in IL-10 production at 0.3 – 3.0 µg/ml (*$p \leq$ 0.05) compared to untreated controls (5.34 ± 3.34 pg/ml). The maximal response of 48.42 ± 3.21 pg/ml was elicited by 0.3 µg/ml α-MSH, although this was not significantly different compared to 3.0 µg/ml (43.83 ± 2.24 pg/ml, $p \leq$ 0.05; Figure 3.34). IL-10 release was subsequently evaluated at 24 h, in order to determine if this induction was maintained over a longer time frame. At this time-point a different scenario was observed with concentrations of 3.0 and 30.0 µg/ml able to cause detectable IL-10 release. The maximal response at this time-point was elicited by 30.0 µg/ml α-MSH (50.19 ± 4.12 pg/ml).

Given that the pan-melanocortin agonist α-MSH caused increases in IL-10 the MC$_3$ agonist D[TRP]^8-γ-MSH (0.3 - 30.0 µg/ml) was subsequently evaluated prior to stimulation with TNF-α (60.0 pg/ml). The MC$_3$ agonist caused a marked release of IL-10 at 2, 6 and 24 h post stimulation, with concentrations of 3.0 and 30.0 µg/ml proving to be the most effective. D[TRP]^8-γ-MSH (30.0 µg/ml) caused a maximal release of 34.12 ± 2.52 pg/ml of IL-10 at 2 h, compared to untreated controls (1.625 ± 0.919 pg/ml; *$p \leq$ 0.05). At 6 h post stimulation 3.0 and 30.0 µg/ml of D[TRP]^8-γ-MSH significantly increased IL-10 release with a peak of 24.94 ± 2.15 pg/ml at 3.0 µg/ml. At 24 h a concentration-dependent increase in IL-10 release was observed with a maximal release of 37.33 ± 0.83 pg/ml at 3.0 µg/ml (Figure 3.34).
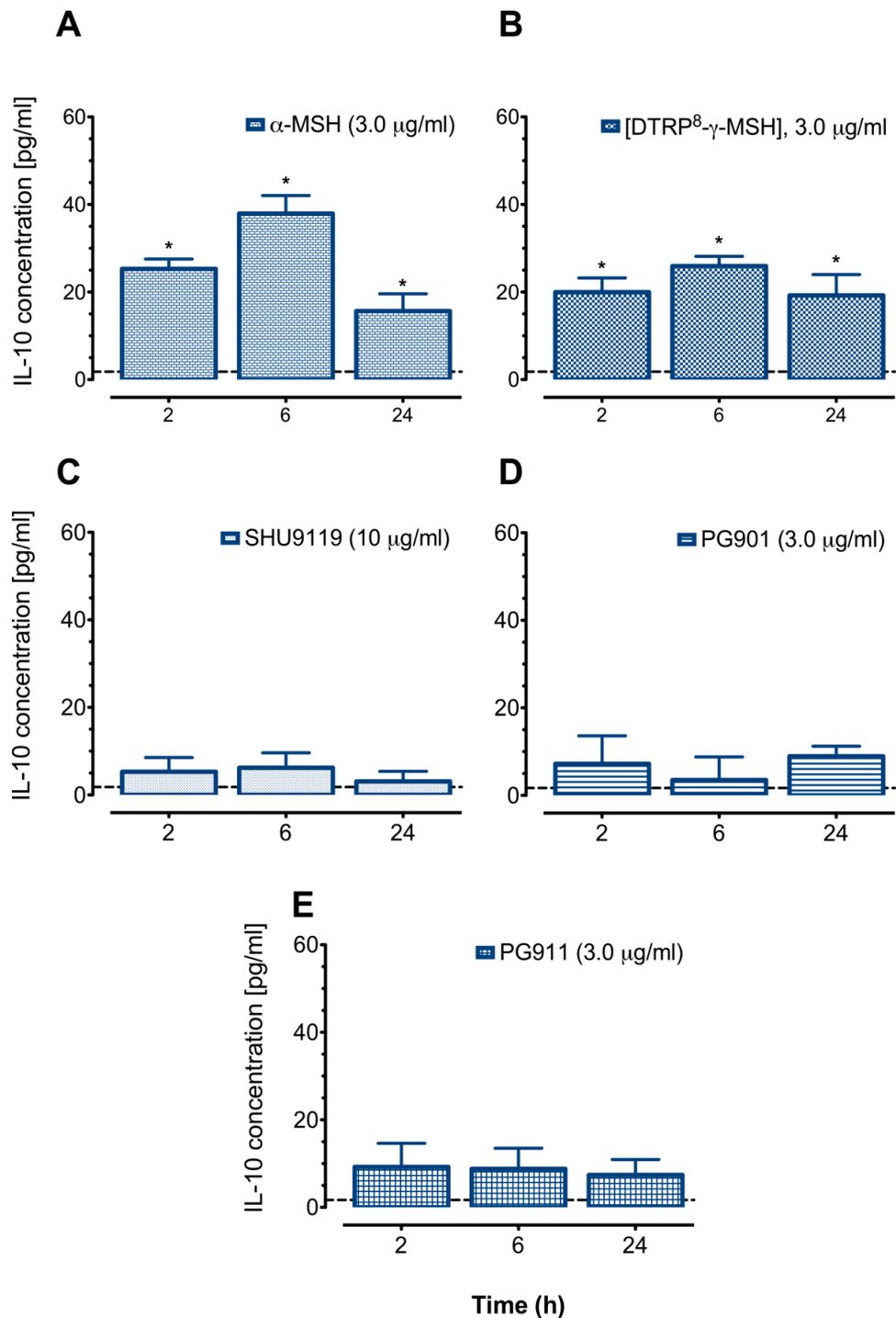
Figure 3.33. Time dependent release of IL-10 from C20/A4 cells following treatment with α-MSH, D[TRP]^8-γ-MSH, SHU9119, PG901 and PG911.

C-20/A4 chondrocytes were treated α-MSH (3.0 μg/ml, Panel A), D[TRP]^8-γ-MSH (3.0 μg/ml; Panel B), SHU9119 (10.0 μg/ml; Panel C), PG901 (3.0 μg/ml; Panel D) or PG911 (3.0 μg/ml; Panel E) and cell-free supernatants collected at 0, 2, 6 and 24 h. IL-10 was then determined by ELISA. Data are presented as Mean± SEM of n=4 independent experiments repeated in triplicate, *$p \leq$ 0.05, vs. Time 0 (dotted line).
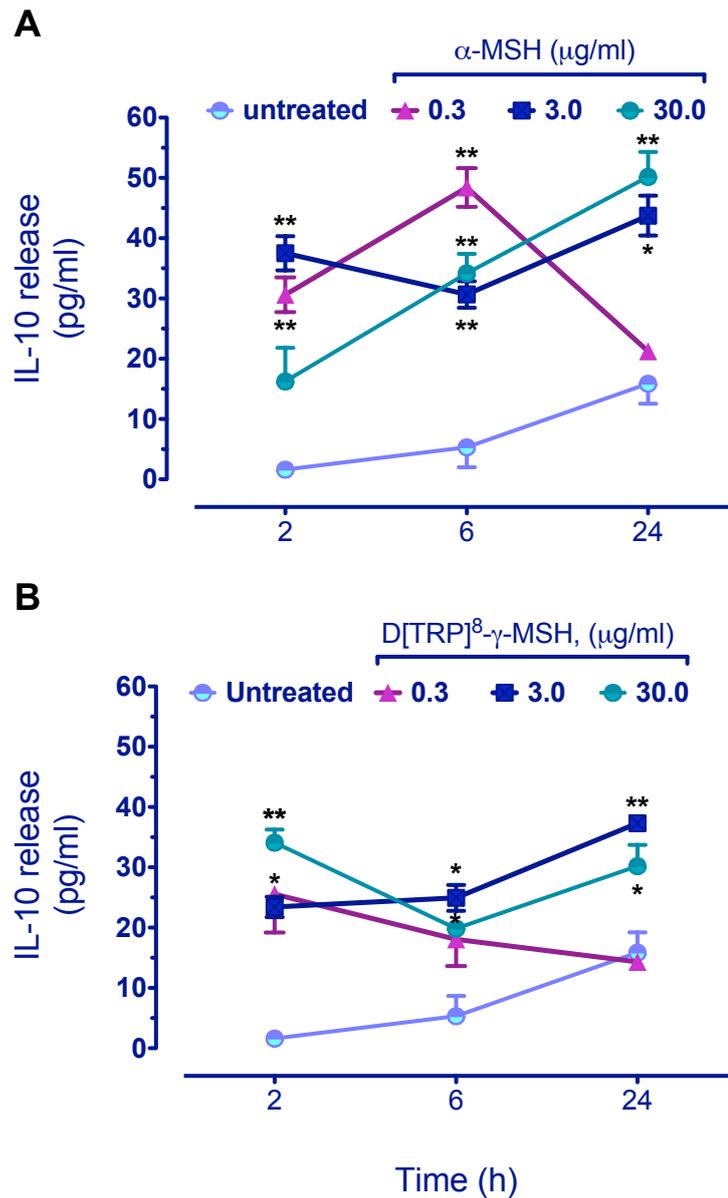
Figure 3.34 Effect of α-MSH and D[TRP]^8-γ-MSH on IL-10 release from TNF-α-activated C-20/A4 chondrocytes.

C-20/A4 chondrocytes were treated with α-MSH or D[TRP]^8-γ-MSH (0.3 – 30.0 μg/ml) for 30 min prior to TNF-α (60.0 pg/ml) stimulation. Cell-free supernatants were collected at for 2, 6 and 24 h and analysed for IL-10 by ELISA. Data are presented as Mean ± SEM of n=4 independent experiments repeated in triplicates. *$p \leq$ 0.05, **$p \leq$ 0.01 vs. untreated control cultures.

3.3.5.3 Determination of HO-1 release from C-20/A4 cells following melanocortin peptide stimulation.

HO-1 release from C-20/A4 chondrocytes treated with vehicle (DMEM), α-MSH or D[TRP]^8-γ-MSH (3.0 μg/ml) alone or in presence of TNF-α (60.0 pg/ml) was investigated via Western Blot and the results are shown in Figure 3.35. C-20/A4 chondrocytes were treated with melanocortin peptides for 6 h either alone or given 30 min prior to stimulation with TNF-α and samples collected 6 h later.

Western Blot analysis identified basal production of HO-1 (0.50 ± 0.04 au), which was significantly increased following treatment with TNF-α (60.0 pg/ml) for 6 h to 1.02 ± 0.01 au ($p \leq$ 0.001). The treatment of chondrocytes with α-MSH (3.0 μg/ml) alone caused a significant 3-fold increase in HO-1 production (1.39 ± 0.04 au; $p \leq$ 0.001), compared to the media-treated controls and a 36 % increase when compared to TNF-α alone ($p \leq$ 0.01). The combination of α-MSH and TNF-α caused a significant increase in HO-1 with a 6-fold increase in HO-1 (2.64 ± 0.14 au, $p \leq$ 0.001) compared to untreated controls and an approximate 3-fold increase when compared to TNF-α-stimulated samples ($p \leq$ 0.001).

A similar observation was made following treatment of the cells with the MC$_3$ agonist D[TRP]^8-γ-MSH (3.0 μg/ml), which caused a 3-fold increase in HO-1 expression, raising it to 1.37 ± 0.02 au ($p \leq$ 0.01) compared to untreated controls and 34 % increase compared to HO-1 production by TNF-α-stimulated chondrocytes ($p \leq$ 0.01). Combination of the MC$_3$ agonist with TNF-α caused the highest increase in HO-1 (2.87 ± 0.12 au, $p \leq$ 0.001) protein with a ~ 7-fold increase compared to media-treated samples, and a ~ 3-fold increase, compared to TNF-α stimulated samples. The expression of α-Tubulin standards was found to be consistent with all treatments. HO-1 production was normalized to the standard expression in that reaction (Figure 3.35 A).
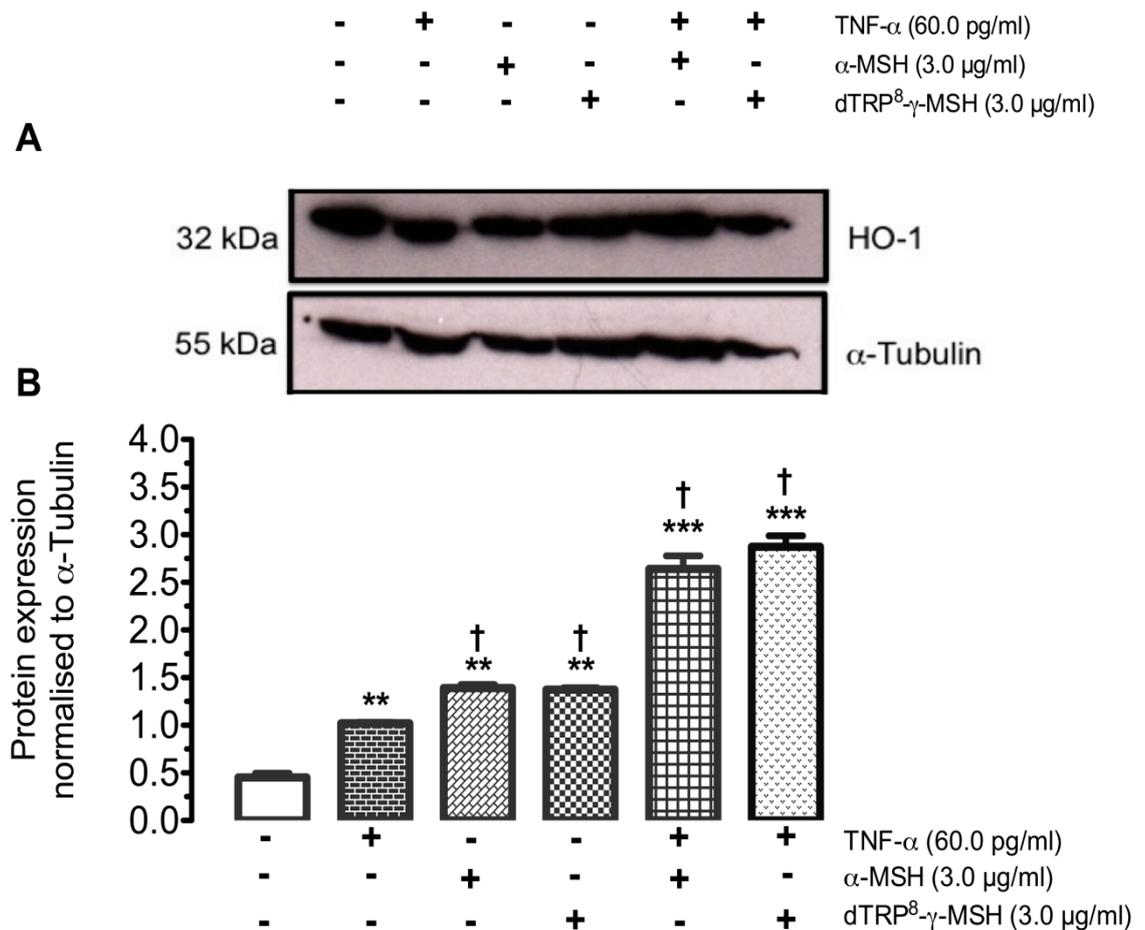
Figure 3.35. Effect of α-MSH and D[TRP]^8-γ-MSH on HO-1 protein levels in human C-20/A4 chondrocytic cell line.

Cultures of C-20/A4 chondrocytes were treated for 6 h with: Lane 1: DMEM medium, Lane 2: TNF-α (60 pg/ml), Lane 3: α-MSH (3.0 μg/ml), Lane 4: D[TRP]^8-γ-MSH (3.0 μg/ml), Lane 5: α-MSH + TNF-α (60 pg/ml), Lane 6: D[TRP]^8-γ-MSH (3.0 μg/ml) + TNF-α (60.0 pg/ml). Bands with sizes corresponding to HO-1 (32 kDa) and α-tubulin (55 kDa) were detected by Western blotting. The image is representative of four individual experiments (Panel A). Comparison of densitometrically quantified HO-1 expression by human C-20/A4 cell-lines is shown in arbitrary units (Panel B). Data are presented as Mean ± SEM of n=4 experiments. $^\dagger p \leq 0.01$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ vs. cultures treated with TNF-α alone.

3.3.6   Effect of melanocortin peptides on chondrocyte apoptosis.

3.3.6.1   Effect of α-MSH and D[TRP]^8-γ-MSH on basal caspase 3/7 activity

Substantial evidence supports that chondrocyte death, due to inflammation and/or injury is an important risk factor predisposing to osteoarthritis and one that exacerbates the disease progression, as chondrocytes are unable to reproduce and compensate for the lost cells (Sharif *et al.,* 2004; Lopez-Armada *et al.,* 2006). We have shown that melanocortins do not cause any statistically significant increase in the production of pro-inflammatory cytokines from C-20/A4 (Table 3.3), and in order to investigate their effect on chondrocyte apoptosis, we employed Caspase-Glo®-3/7 assay to measure any changes in caspase-3/7 activity following treatment.

C-20/A4 chondrocytes were treated with vehicle (DMEM), α-MSH or D[TRP]^8-γ-MSH (0.1 – 30.0 µg/ml) for 6 h and caspase-3/7 activity was determined according to the manufacturer's instructions (Promega UK Ltd., UK). Both α-MSH and D[TRP]^8-γ-MSH (0.1 – 30.0 µg/ml), were did not activate caspase-3/7 apoptotic pathway (Figure 3.36), however α-MSH caused a moderate non-significant inhibition of the basal level of activity of the enzymes.
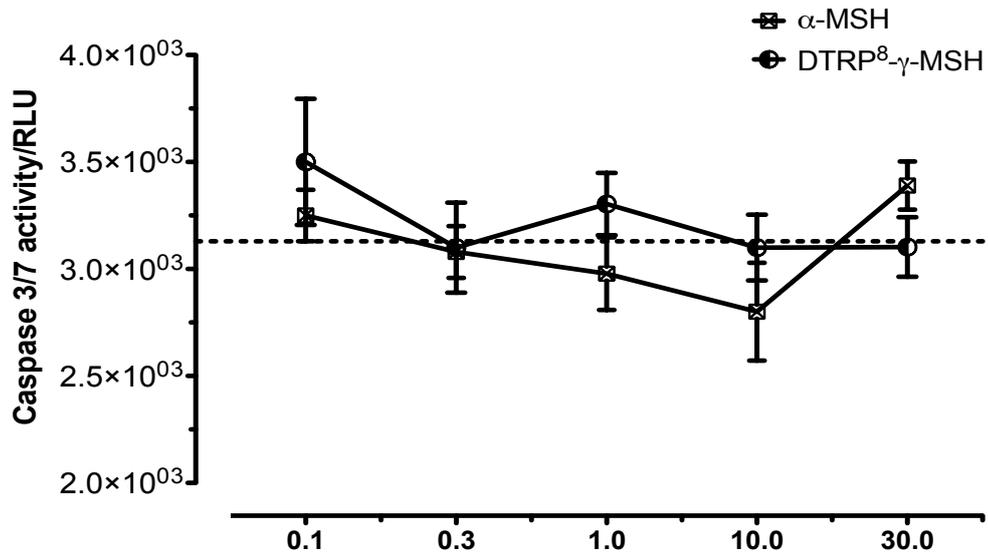
Figure 3.36. The effect of α-MSH and D[TRP]^8-γ-MSH on caspase 3/7 activity of untreated C-20/A4 chondrocytes.

C-20/A4 chondrocytes were plated at 2.0 x 10^4 cells/well, and treated with DMEM or D[TRP]^8-γ-MSH (0.1 – 30.0 µg/ml) for 30 min prior to stimulation with TNF-α (60.0 pg/ml) for 6 h. Caspase-3/7 activity was determined by Caspase-3/7 Glo Assay (Promega, UK) Dotted line symbolizes Caspase 3/7 activity following PBS treatment (control). Data are presented as Mean ± SEM of n=4 experiments, assessed in triplicates.

3.3.6.2 Melanocortin peptides inhibit caspase-3 production, caspase-3/7 activity and cell death of TNF-α-activated C-20/A4 chondrocytes.

C-20/A4 chondrocytes were treated with TNF-α (60.0 pg/ml) to establish levels of caspase-3 activity (Figure 3.37). Western blot analysis against cleaved (activated) caspase-3 (Asp-175) showed 0.52 ± 0.045 au basal production (DMEM) of cleaved caspase-3, which upon stimulation with TNF-α led to 0.65 ± 0.24 au thereby representing a significant 24 % increase in production ($p \leq 0.05$). Upon pre-treatment of the cells with MC pan-agonist α-MSH and the selective MC$_3$ agonist D[TRP]^8-γ-MSH, there was significant reduction in the synthesis of activated caspase-3 compared to the levels caused by TNF-α, with a 50 % (0.32 ± 0.012 au) and 42 % (0.37 ± 0.03) reduction for α-MSH (3.0 µg/ml) and D[TRP]^8-γ-MSH (3.0 µg/ml) respectively ($p \leq 0.01$).

In order to investigate the involvement of the specific melanocortin receptors, SHU9119 (10.0 µg/ml) was utilized to specifically block MC$_3$. When used in conjunction with α-MSH it did not block the effect of the peptide however, the antagonist did cause a slight (~8.7 %; n.s. $p > 0.05$) synergistic inhibition in the production of activated caspase-3 (0.27 ± 0.02 au) compared to the effect of α-MSH + TNF-α alone.

The opposite effect was observed when SHU9119 was given in conjunction with the selective MC$_3$ agonist D[TRP]^8-γ-MSH, whereby it markedly hindered D[TRP]^8-γ-MSH from activating its receptor (Figure 3.37). The combination led to significant ~25 % increase in cleaved caspase-3 production (0.47 ± 0.01 au; $p \leq 0.01$) compared to the effect of D[TRP]^8-γ-MSH without the antagonist (0.37 ± 0.3 au).

Cell viability was evaluated using MTT proliferation assay (Figures 3.38, 3.39). C-20/A4 chondrocytes were treated with DMEM, α-MSH or D[TRP]^8-γ-MSH (0.1 – 30.0 µg/ml) ± TNF-α (60.0 pg/ml) for 6 h with cell viability determined via MTT assay and caspase-3/7 activity determined by Caspase-3/7® Glo Assay. TNF-α (60.0 pg/ml) caused a 26 % cell death as determined by MTT Assay, which was alleviated in a concentration-dependent manner by treatment with the peptides;

maximal inhibition of cell death was achieved at 1.0 and 3.0 µg/ml of α-MSH, and 3.0 and 10.0 µg/ml D[TRP]^8-γ-MSH ($p \leq$ 0.01).

Treatment of the C-20/A4 chondrocytes with TNF-α increased caspase-3/7 activity 5.7-fold compared to DMEM, but was attenuated in the presence of α-MSH and D[TRP]^8-γ-MSH in a concentration-dependent manner ($p \leq$ 0.01; Figure 3.38). Pre-treatment of cells with the MC$_{3/4}$ antagonist SHU9119 fully reversed the effect of D[TRP]^8-γ-MSH, but had no effect on α-MSH effect on these parameters.
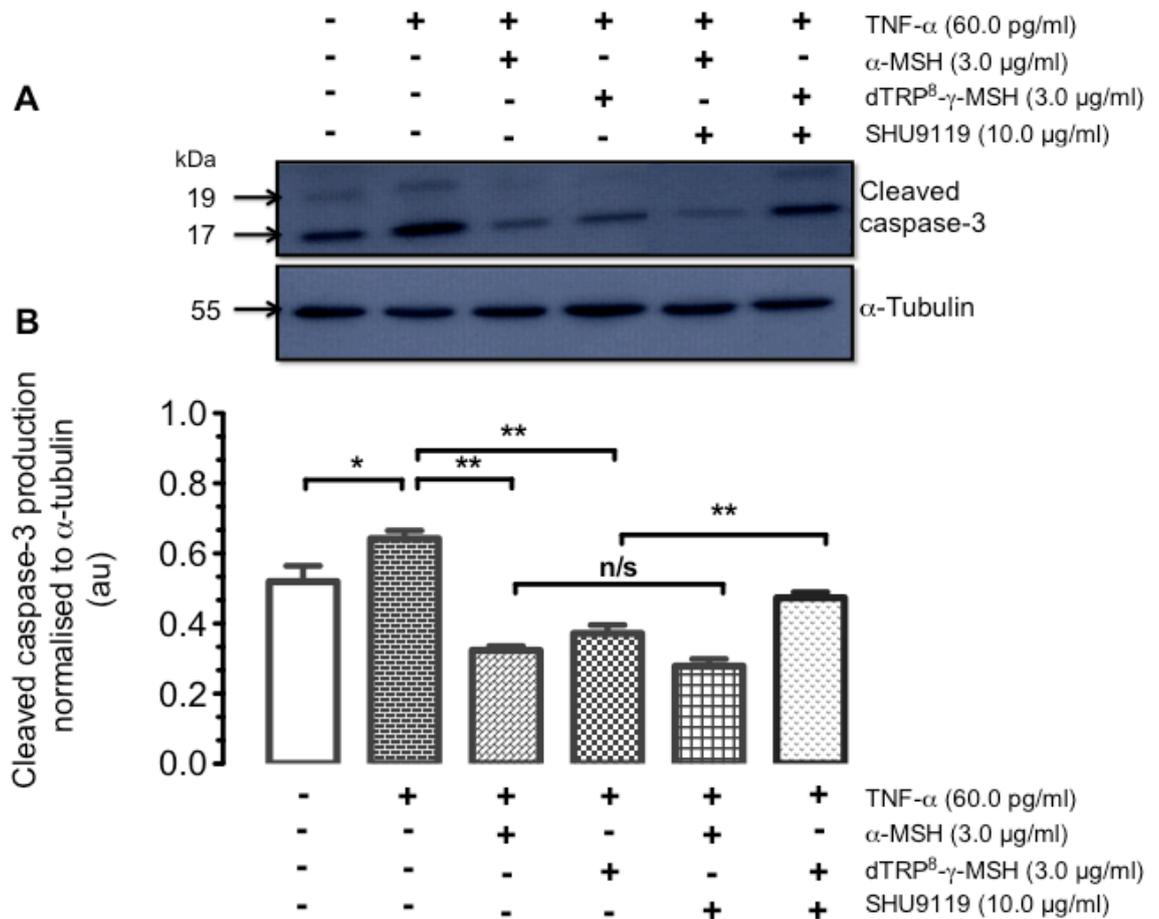
Figure 3.37. Western Blot detection and densitometric quantification of Cleaved caspase-3 protein levels in human C-20/A4 chondrocytic cell line.

Cultures of C-20/A4 chondrocytes were treated for 6 h with: Lane 1: DMEM medium, Lane 2: TNF-α (60 pg/ml), Lane 3: α-MSH (3.0 μg/ml), Lane 4: D[TRP]^8-γ-MSH (3.0 μg/ml), Lane 5: α-MSH + TNF-α (60 pg/ml), Lane 6: D[TRP]^8-γ-MSH (3.0 μg/ml) + TNF-α (60 pg/ml). Bands with sizes corresponding to Cleaved caspase-3 (Asp175; 17, 19 kDa) and α-tubulin (55 kDa) were detected by Western blotting. The image is representative of 4 individual experiments (Panel A). Comparison of densitometrically quantified Cleaved caspase-3 (Asp175) expression by human C-20/A4 cell-lines is shown in arbitrary units (au, Panel B). Data are presented as Mean ± SEM of n=4 experiments: **$p \leq 0.01$, ***$p \leq 0.001$ vs. respected control.
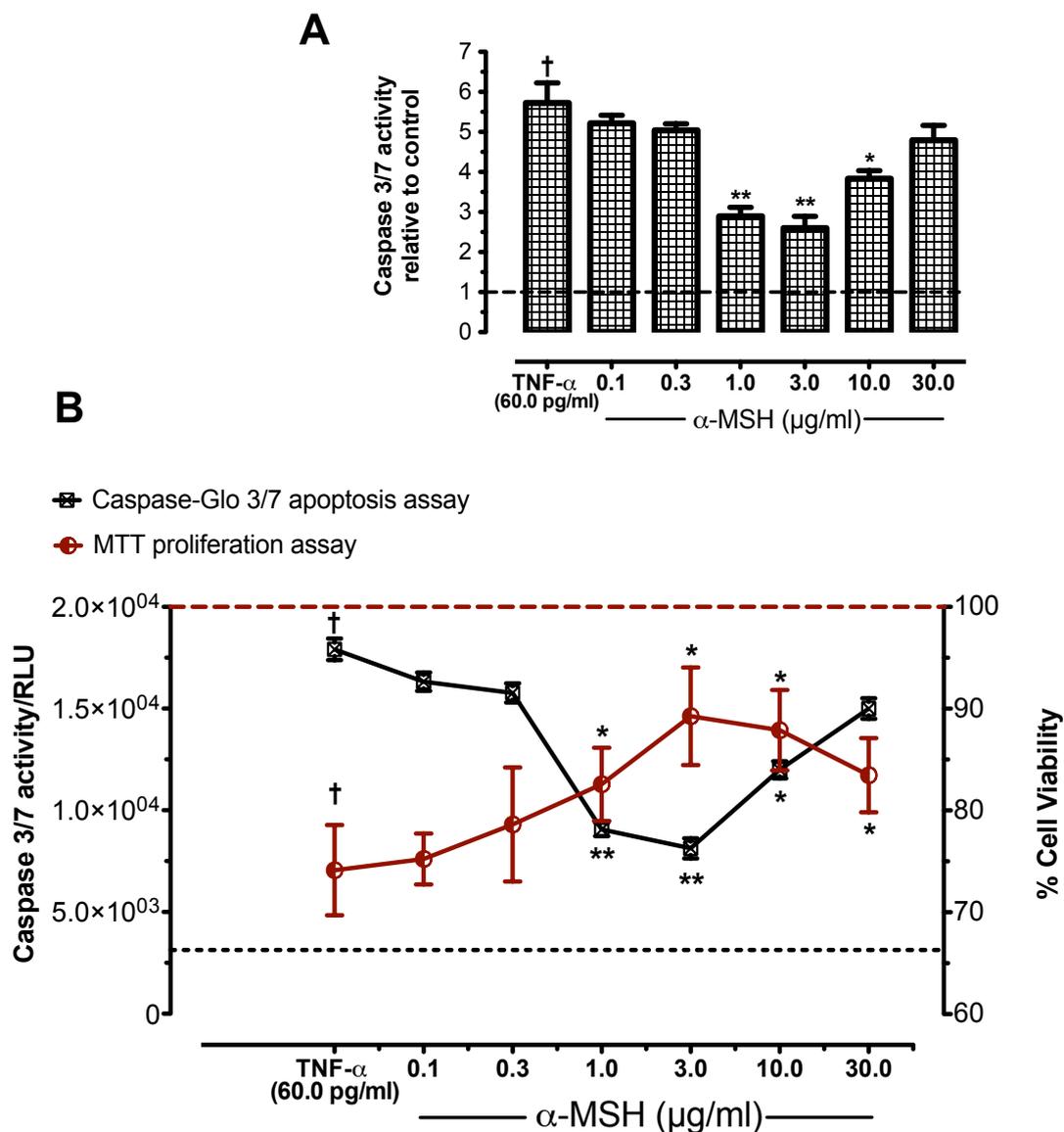
Figure 3.38. **The effect of α-MSH on cell viability and caspase 3/7 activity on TNF-α-activated C-20/A4 chondrocytes.**

C-20/A4 chondrocytes were plated at 2.0 x 10^4 cells/well, and treated with PBS or α-MSH (0.1 – 30.0 μg/ml) for 30 min prior to stimulation with TNF-α (60.0 pg/ml) for 6 h. Cell viability was determined via MTT reduction assay (Panel B) and caspase-3/7 activity determined by Caspase-3/7 Glo Assay (Panels A and B). Dashed (red) line represents control sample cell viability – untreated cells as determined by MTT (100%); Dotted line symbolizes Caspase 3/7 activity following DMEM treatment (control). Data are presented as Mean ± SEM of n=4 experiments, assessed in triplicate. *p≤ 0.05, **p≤ 0.01, **p≤ 0.01 vs. TNF-α-treated controls; †p≤ 0.01 vs. untreated controls.
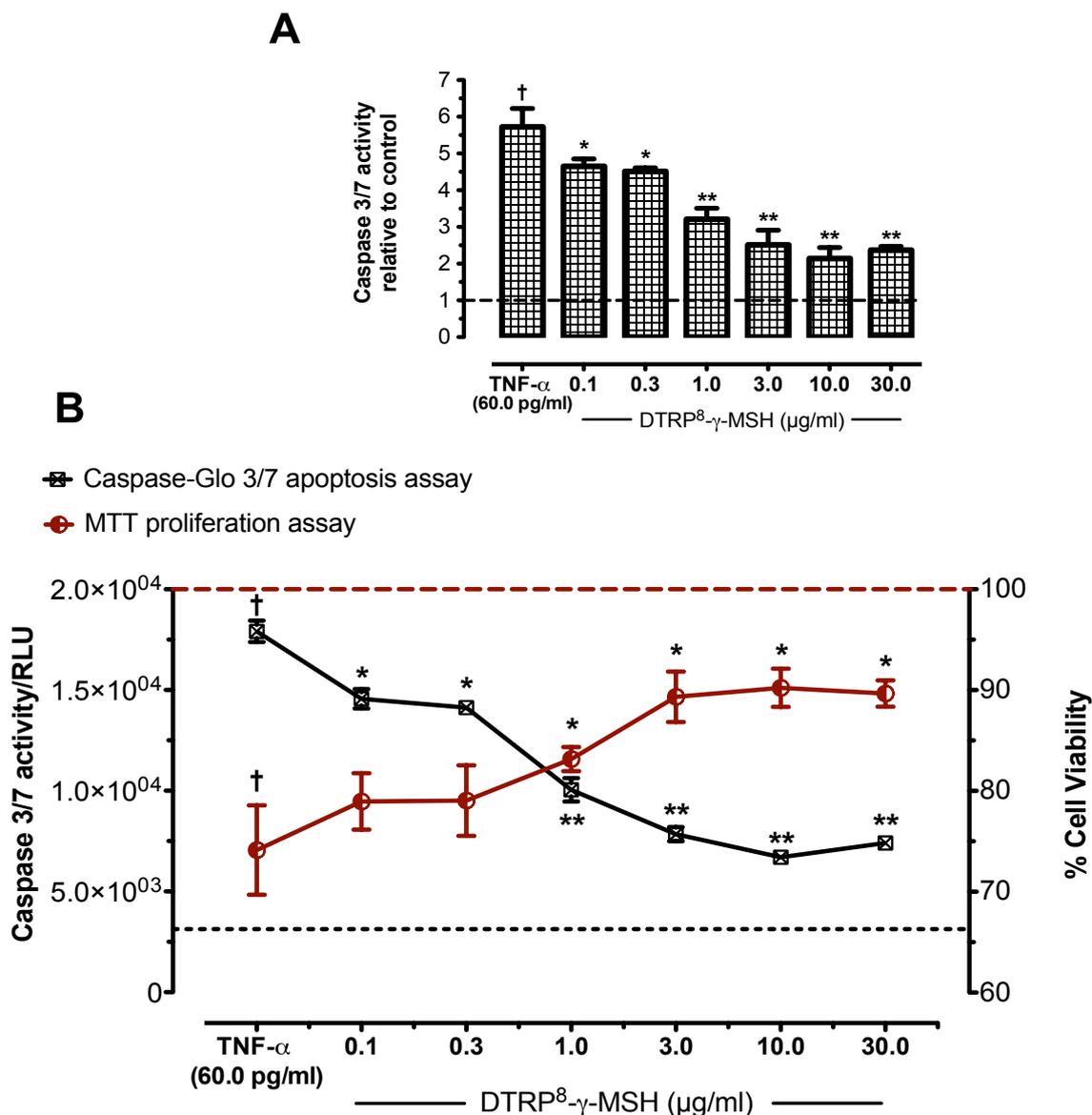
Figure 3.39. The effect of D[TRP]^8-γ-MSH on cell viability and caspase 3/7 activity on TNF-α-activated C-20/A4 chondrocytes.

C-20/A4 chondrocytes were plated at 2.0 x 10^4 cells/well, and treated with PBS or D[TRP]^8-γ-MSH (0.1 – 30.0 µg/ml) for 30 min prior to stimulation with TNF-α (60.0 pg/ml) for 6 h. Cell viability was determined via MTT reduction assay (Panel A) and caspase-3/7 activity determined by Caspase-3/7 Glo Assay (Panel A and B). Dashed (red) line represents control sample cell viability – untreated cells (100%); Dotted line symbolizes Caspase 3/7 activity following PBS treatment (control). Data are presented as Mean ± SEM of n=4 experiments, assessed in triplicate. *p≤ 0.05, **p≤ 0.01, ***p≤ 0.001 vs. vs. TNF-α-treated controls; †p≤ 0.01 vs. untreated controls.
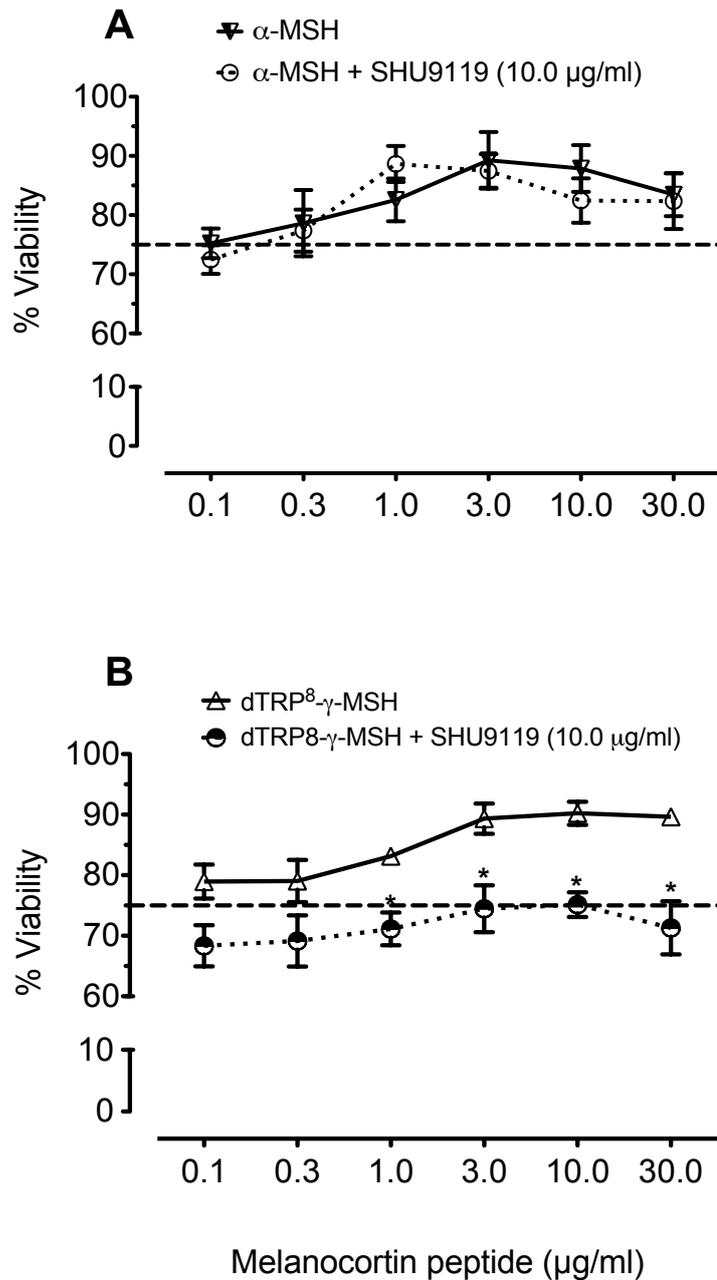
Figure 3.40. The effect of α-MSH and D[TRP]^8-γ-MSH on cell viability following TNF-α activation.

C-20/A4 chondrocytes were plated at 2.0 x 10^4 cells/well, and pre-treated with a-MSH/D[TRP]^8-γ-MSH (0.1 – 30.0 µg/ml) ± SHU9119 (10.0 µg/ml) for 30 min prior to stimulation with TNF-α (60.0 pg/ml) for 6 h. Cell viability was determined via MTT reduction assay. Dashed line represents TNF-a (60.0 pg/ml) treated control cell viability – 74.13%. Data are presented as Mean ± SEM of n=4 experiments, assessed in triplicate. *$p \leq$ 0.05, **$p \leq$ 0.01, compared to respective control.

3.3.7   Effect of hypo-osmolarity on C-20/A4 chondrocyte function.

Cartilage hydration plays an important role in the physiology of healthy chondrocytes. Highly hydrated proteoglycans inflate the cartilage tissue and thus it confers an ability to resist compressive forces (Hall, 1998). However, during osteoarthritis, matrix metalloproteinases are significantly over-expressed, driving the major degradation of collagen that occurs during OA (Kevorkian *et al*., 2004, Kobayashi *et al*., 2005). The damaged collagen network allows the charged proteoglycans to attract more water, which leads to dilution of the charged ions in the extracellular space and misbalance of osmolarity compared to the chondrocytic cytoplasm, thereby increased water-flow into the cell and altered metabolism (Maroudas, 1976, Bush and Hall, 2001a, Bush and Hall, 2005). As it is very difficult to mimic these conditions *in situ* and *in vivo,* the effect of hypotonic media (140 mOsm:H_2O) on chondrocyte function was investigated in C-20/A4 chondrocyte system. The production of pro-inflammatory cytokine production and expression of matrix metalloproteinases was investigated following treatment of the chondrocytes with hypo-osmotic (140 mOsm) DMEM culture media.

3.3.7.1   Effect of hypo-osmolarity on pro-inflammatory cytokines release and MMPs expression in C-20/A4 chondrocytes.

C-20/A4 chondrocytes stimulated with TNF-α (60 pg/ml) showed increased pro-inflammatory cytokine expression and release (IL-1β, IL-6, IL-8) and significant rise in *MMP1* and *MMP13*. Here, an *in vitro* model, was designed to mimic the hypo-osmolarity in osteoarthritic cartilage in order to investigate its effects on the production of the catabolic pro-inflammatory cytokines IL-6 and IL-8, and the detrimental collagenases 1 and 3.

C-20/A4 chondrocytes were grown in hypotonic media (140 mOsm) for 0, 24 and 72 h and *IL6* and *IL8* expression determined using semi-quantitative PCR amplification reactions with the appropriate primers (Table 2.9). PCR products were visualised on 2 % agarose gel and the results were normalised to internal control *β-actin* (Figure 3.41 A). The resulting bands were analysed by densitometry (Figure 3.41 B). There was a significant increase in *IL6* and *IL8*

mRNA levels. Cells incubated for 24 h in 140 mOsm DMEM media released 37-fold more *IL6* compared to control cultures and with increasing the time-periods to 72 h, the expression of *IL6* increased to 42-fold compared to cells incubated in normal 280 mOsm DMEM (Figure 3.41). Similarly, *IL8* expression was significantly increased following incubation with hypo-osmotic solution. The treatment of C-20/A4 cells with hypo-osmotic solution for 24 h caused a 8-fold up-regulation of *IL8* expression, which continued to increase to 10-fold at 72 h post-stimulation.

Changes in protein levels of IL-6 and IL-8 were investigated following incubation of the cells in 140 mOsm DMEM for 24 and 72 h by ELISA (Figure 3.42). C-20/A4 cells were incubated with 140 mOsm DMEM media for 24 or 72 h. Cell-free supernatants were collected and analysed for IL-6 and IL-8 production by commercially available IL-6 and IL-8 ELISAs according to manufacturer's instructions (R&D Systems, UK). There was a significant time-dependent increase in the concentration of IL-6 as compared to time 0. C-20/A4 chondrocytes responded to decreased osmolarity of the extracellular space (DMEM) with a release of 150 ± 12.66 pg/ml and 192.34 ± 7.83 pg/ml of IL-6 following incubation with the hypo-osmotic medium for 24 and 72h respectively ($p \leq$ 0.001 for both values) compared to time 0 (21.09 ± 18.5 pg/ml). Similar observations were made for IL-8 production following incubation with 140 mOsm DMEM media for 24 and 72h, which yielded 394.32 ± 15.60 pg/ml and 376.10 ± 11.38 pg/ml respectively ($p \leq$ 0.001), compared to the untreated control cells (72.65 ± 4.78 pg/ml). There was slight but insignificant reduction in IL-8 production between 24 and 72 h post stimulation (Figure 3.42).
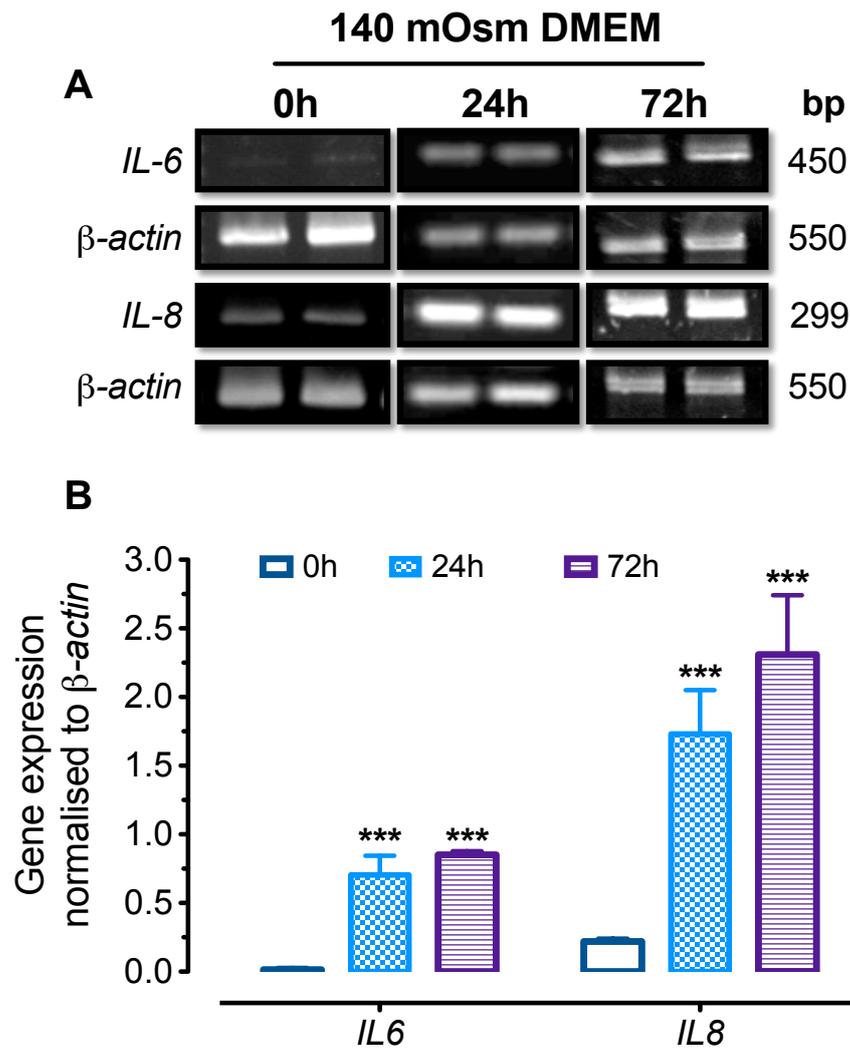
Figure 3.41. Densitometric quantification of *IL-6* and *IL-8* expression in C-20/A4 cells incubated in hypotonic (140 mOsm) DMEM media for 24 h and 72 h.

C-20/A4 chondrocytes were stimulated with hypotonic media (140 mOsm) at time 0 and total RNA extracted at 0 h, 24 h and 72 h post stimulation. PCR amplification with the respective primers for *IL6* and *IL8* was used to detect and quantify gene expression on 2 % agarose gels in triplicates with *β-actin* used as internal control (Panel A). Comparison of densitometrically quantified *IL-6* (Panel B) and *IL-8* (Panel C) shown in arbitrary units, each value normalized to the respective *β-actin* expression. Data are presented as Mean ± SEM of n=4 independent experiments ***$p \leq$ 0.001).
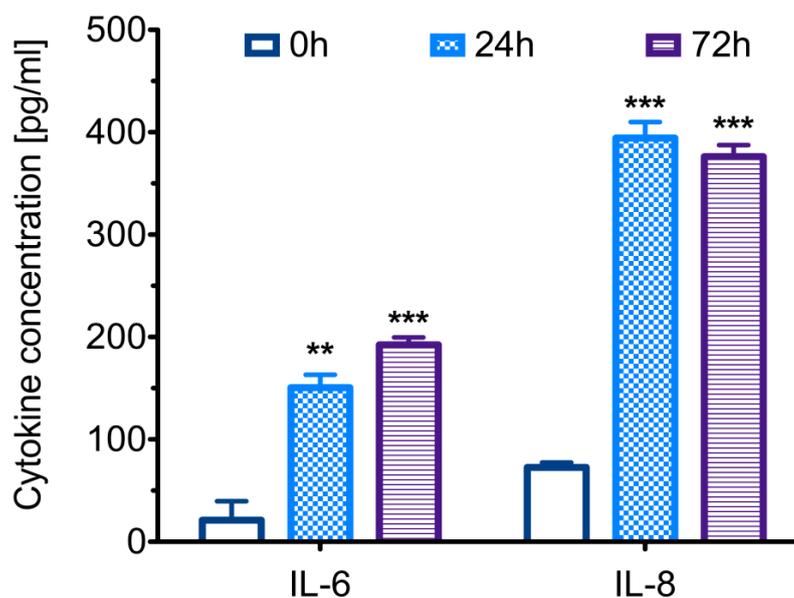
Figure 3.42 Time dependent effect of hypo-osmotic (140 mOsm) DMEM media on IL-6 and IL-8 protein release.

C-20/A4 chondrocytes were treated with hypotonic media (140 mOsm) at time 0 and cell-free supernatants were collected at 0, 24 and 72 h post stimulation. Supernatants were then analysed by commercially available ELISA for IL-6 and IL8 production. Data are presented as Mean ± SEM of n=3 independent experiments repeated in triplicate, **$p \leq 0.01$, ***$p \leq 0.001$) vs. Time 0.

The effect of hypotonic media on *MMP1*, *MMP3* and *MMP13* expression was determined using PCR amplification reactions with the appropriate primers (Table 2.9). The PCR products were visualised on 2 % agarose gel and the results were normalised to internal control *β-actin* (Figure 3.43 A). Densitometric analysis showed that upon incubation of C-20/A4 chondrocytes with 140 mOsm DMEM media for 24 h and 72 h, *MMP1* expression was significantly elevated with 0.49 ± 0.12 au (2.3-fold increase; $p \leq 0.05$) at 24 h and a 4-fold increase at 72 h (0.932 ± 0.0263 au; $p \leq 0.001$ vs. Time 0 h). Interestingly, *MMP3* expression was increased slightly following 72 h compared to non-treated cells (0.714 ± 0.04 au, 1.23-fold; $p \leq 0.05$ vs. Time 0), whilst no significant changes were observed at 24 h. *MMP13*, a collagenase that is expressed in neither non-stimulated C-20/A4 cells, (as shown on Figure 3.43 B), nor in healthy human cartilage, was significantly up-regulated to 0.557 ± 0.058 au after 24 h incubation in 140 mOsm DMEM ($p \leq 0.001$), and expression was increased following 72 h (0.642 ± 0.01 au; $p \leq 0.001$ vs. Time 0).
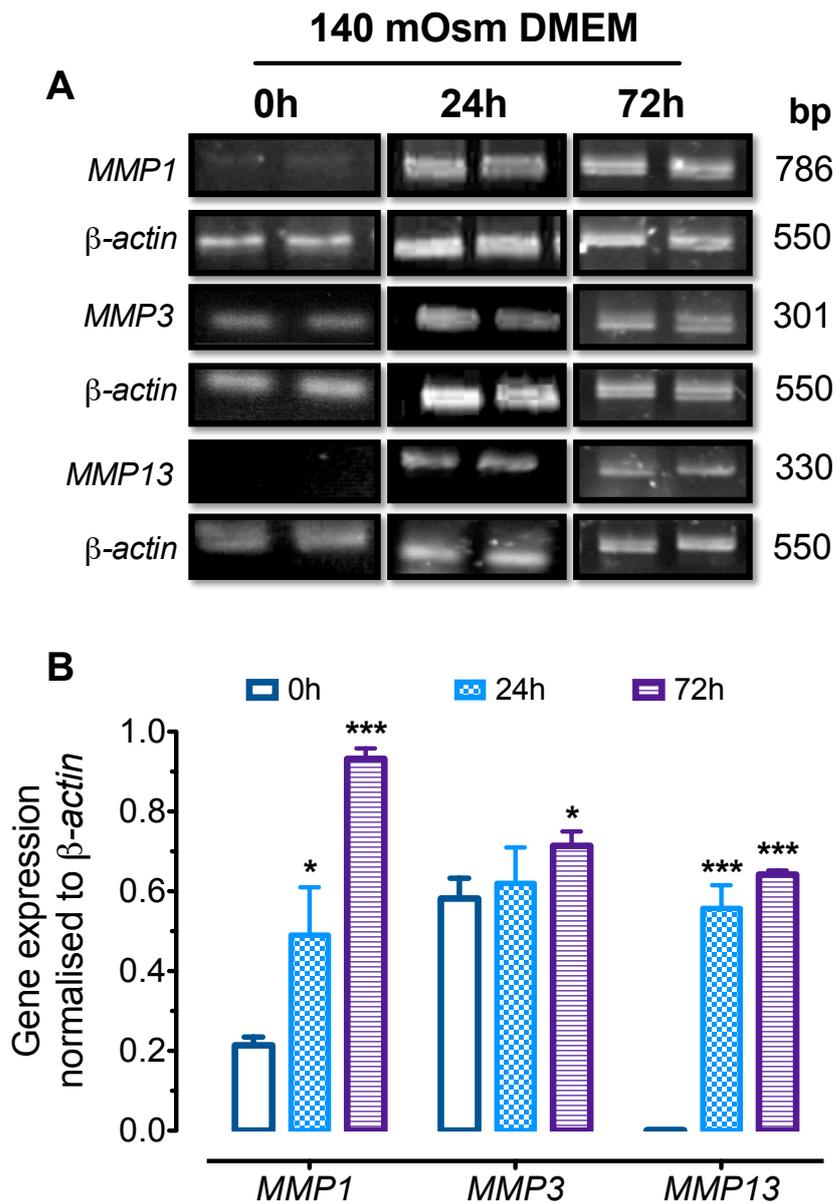
140 mOsm DMEM

Figure 3.43. Densitometric quantification of _MMP1, MMP3_ and _MMP13_ expression in C20/A4 cells treated with 140 mOsm DMEM media.

C20/A4 chondrocytes were stimulated with hypotonic media (140 mOsm) at time 0 and total RNA extracted 0-72 h post stimulation. PCR amplification with the respective primers for _MMP1_, _MMP3_ and _MMP13_ was used to detect and quantify gene expression on 2 % agarose gels in duplicates with _β-actin_ used as internal control (Panel A). Comparison of densitometrically quantified _MMP1_, _MMP3_ and _MMP13_ expression induced by hypotonic saline (140 mOsm:H_2O, Panel B) shown in arbitrary units, each value normalized to the respective _β-actin_ expression. Data is presented as Mean± SEM of n=4 independent experiments *$p \leq$ 0.05, ***$p \leq$ 0.001).

3.3.7.2 Attenuation of extracellular hypo-osmolarity-induced matrix metalloproteinases production by melanocortin peptides α-MSH and D[TRP]^8-γ-MSH.

In order to investigate different aspects of the anti-inflammatory properties of the melanocortin peptides α-MSH and D[TRP]^8-γ-MSH, their effect was evaluated on the up-regulated expression of catabolic proteinases caused by the hypo-osmotic challenge.

C-20/A4 cells were incubated in 140 mOsm DMEM for 24 h + α-MSH (3.0 µg/ml) or D[TRP]^8-γ-MSH (3.0 µg/ml). Total RNA was extracted and *MMP1*, *MMP3* and *MMP13* gene expression was analysed by semi-quantitative PCR (Figure 3.44 A) with the specific primers for the amplification of the individual genes listed in Table 2.9. Following treatment for 24 h, α-MSH failed to cause any significant inhibition of the expression of *MMP1, MMP3* and *MMP13*. Similarly, D[TRP]^8-γ-MSH at 3.0 µg/ml failed to cause any detectable change in the expression of *MMP3*, however, the MC$_3$ agonist reduced the transcription of MMP1 (from 0.49 ± 0.12 au to 0.25 ± 0.05 au) and *MMP13* (from 0.56 ± 0.06 au to 0.11 ± 0.01 au), representing 49 % and 80 % inhibition respectively (Figure 3.44 B).
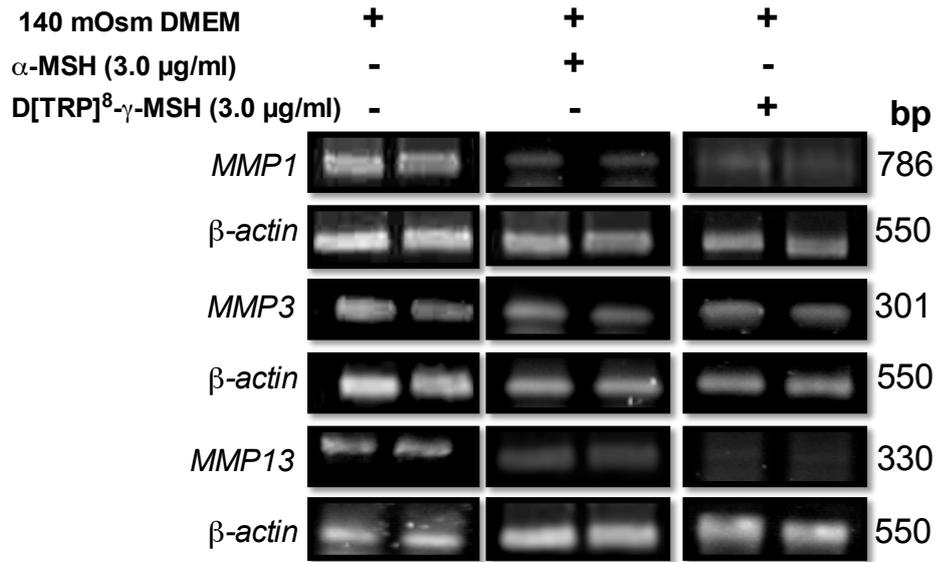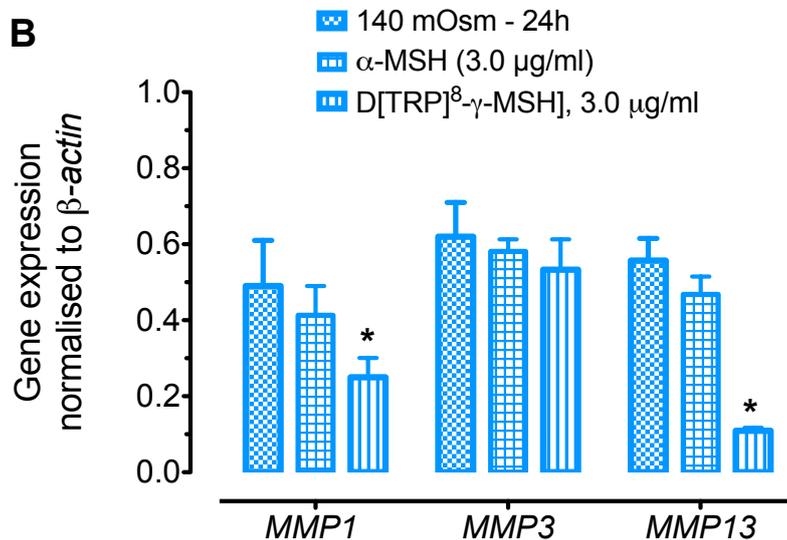
Figure 3.44. Effect of melanocortins on *MMP1, MMP3* and *MMP13* expression in C-20/A4 cells treated with 140 mOsm DMEM for 24 h measured by densitometry.

C-20/A4 chondrocytes were pre-treated with α-MSH or D[TRP]^8-γ-MSH prior to stimulated with hypotonic media (140 mOsm) at time 0 and total RNA extracted 24 h post stimulation. PCR amplification with the respective primers for *MMP1*, *MMP3* and *MMP13* was used to detect and quantify gene expression on 2 % agarose gels in duplicates with *β-actin* used as internal control (Panel A). Comparison of densitometrically quantified *MMP1*, *MMP3* and *MMP13* expression induced by hypotonic saline (140 mOsm; Panel B) shown in arbitrary units, each value normalized to the respective *β-actin* expression. Data are presented as Mean ± SEM of n=3 independent experiments *$p \le 0.05$).

3.4 Effect of pro-inflammatory stimuli on primary bovine chondrocytes function.

3.4.1 Detection of pro-inflammatory cytokines release following primary chondrocyte activation.

C-20/A4 cell-line chondrocytes have been shown within this thesis to respond to TNF-α and LPS stimulation leading to the release of pro-inflammatory cytokines. Given these findings, primary bovine articular chondrocytes (freshly extracted, cultured in monolayers) were evaluated to determine whether primary cells responded in a similar fashion, as these have been shown to be a good model for human primary chondrocytes (Kerrigan and Hall, 2005). Isolated bovine articular chondrocytes were cultured and incubated in serum-free 380 mOsm DMEM medium for 24 h prior to stimulation with TNF-α (20.0 – 60.0 pg/ml) and LPS (1.0 – 10.0 μg/ml) for 6 h, cell-free supernatants were collected and analysed for IL-1β, IL-6 and IL-8 by ELISA.

3.4.1.1 Effect of TNF-α and LPS on IL-6 and IL-8 release from bovine chondrocytes.

TNF-α stimulation of primary bovine chondrocytes led to significant increases in IL-6 release (Figure 3.45 A). The pro-inflammatory cytokine (20.0 – 80.0pg/ml) caused a concentration-dependent increase in synthesis and release of IL-6 from the bovine articular chondrocytes with 20.0 pg/ml causing a significant release of 38.54 ± 9.85 pg/ml of IL-6 ($p\leq$ 0.05) compared to untreated controls (3.2 ± 1.38 pg/ml). IL-6 levels doubled following stimulation with 40.0 pg/ml (78.59 ± 13.68 pg/ml, $p\leq$ 0.01). Increasing concentrations of TNF-α led to 174.38 ± 14.6 pg/ml ($p\leq$ 0.001) and 347.07 ± 29.79 pg/ml ($p\leq$ 0.001) of IL-6 production at 60.0 pg/ml and 80.0 pg/ml respectively. LPS (1.0 – 10.0 μg/ml) also caused significant increases in IL-6; however, all concentrations evaluated caused a similar response. A maximal response was achieved following stimulation with LPS (3.0 μg/ml), where 163.23 ± 12.24 pg/ml of IL-6 was released. Both 1.0 and 10.0 μg/ml of LPS had similar effect, causing 156.93 ± 15.35 pg/ml and 159.12 ± 12.24 pg/ml of IL-6 release respectively. The increase caused by LPS at all

concentrations was significantly increased compared to the untreated controls (3.2 ± 1.38 pg/ml; $p \leq 0.001$; Figure 3.45 B).

Following the determination of IL-6, IL-8 levels from stimulated bovine chondrocytes were evaluated. TNF-α (20.0 – 80.0 pg/ml) caused a bell-shaped concentration dependent effect, peaking at 40.0 pg/ml and 60.0 pg/ml with 46.67 ± 13.65 pg/ml ($p<0.05$) and 45.56 ± 2.94 pg/ml ($p \leq 0.01$) respectively, whilst higher concentrations of TNF-α (80.0 pg/ml) caused lower levels of IL-8 release (28.89 ± 3.9 pg/ml ($p \leq 0.01$; Figure 3.45 C). LPS (1.0 – 10.0 μg/ml) caused a similar response with 22.33 ± 3.1 pg/ml ($p \leq 0.01$) and 12.29 ± 1.98 pg/ml ($p \leq 0.05$) IL-8 release for 3.0 μg/ml and 10.0 μg/ml respectively, however, 1.0 μg/ml of LPS failed to induce any significant increase in IL-8 compared to control values ($p> 0.05$; Figure 3.45 B).

3.4.1.2 Effect of TNF-α and LPS on IL-1β and MCP-1 release from bovine chondrocytes.

Treatment of bovine chondrocytes with TNF-α (20.0 – 80.0 pg/ml) caused a bell shaped concentration-dependent effect on IL-1β release following 6 h incubation, peaking at 60.0 pg/ml with 21.87 ± 0.2 pg/ml ($p \leq 0.05$) compared to untreated controls (0.82 ± 0.06 pg/ml; Figure 3.46 A). Lower concentrations of TNF-α caused 17.06 ± 0.65 pg/ml and 17.40 ± 0.23 pg/ml IL-1β release for 20.0 and 40.0 pg/ml respectively ($p \leq 0.05$). Similar response was observed following stimulation with the highest tested concentration 80.0 pg/ml of TNF-α leading to 17.92 ± 0.26 pg/ml IL-1β released ($p \leq 0.05$). LPS stimulation also activated the bovine chondrocytes to produce IL-1β at all concentrations tested (Figure 3.46 B), with a maximal release following stimulation with 3.0 μg/ml, causing a release of 20.8 ± 3.8 pg/ml IL-1β ($p \leq 0.05$). LPS at 1.0 and 10.0 μg/ml led to significant 17.9 ± 1.2 pg/ml and 19.8 ± 2.9 pg/ml IL-1β release from the chondrocytes compared to control ($p \leq 0.05$).

Similarly to the effect on IL-1β, IL-6 and IL-8, TNF-α (20.0 – 80.0 pg/ml) and LPS 1.0 – 10.0 μg/ml) caused significant production of MCP-1 (Figure 3.46 C). TNF-α caused a maximal response at 60.0 pg/ml with 159.44 ± 13.29 pg/ml ($p \leq 0.001$), whilst 20.0 pg/ml was unable to exhibit any effect on the production of MCP-1,

but when the concentration was doubled to 40.0 pg/ml, there was a significant elevation in MCP-1 (79.86 ± 25.12 pg/ml; $p \leq$ 0.001) compared to untreated controls.

When primary bovine cells were treated for 6 h with LPS (1.0 – 10.0 μg/ml) there was a significant increase in MCP-1, with a maximal production of 108.05 ± 4.2 pg/ml at 3.0 μg/ml LPS. All other concentrations caused very similar amount of MCP-1 production ($p \leq$ 0.001 for all doses of LPS used; Figure 3.46 D).
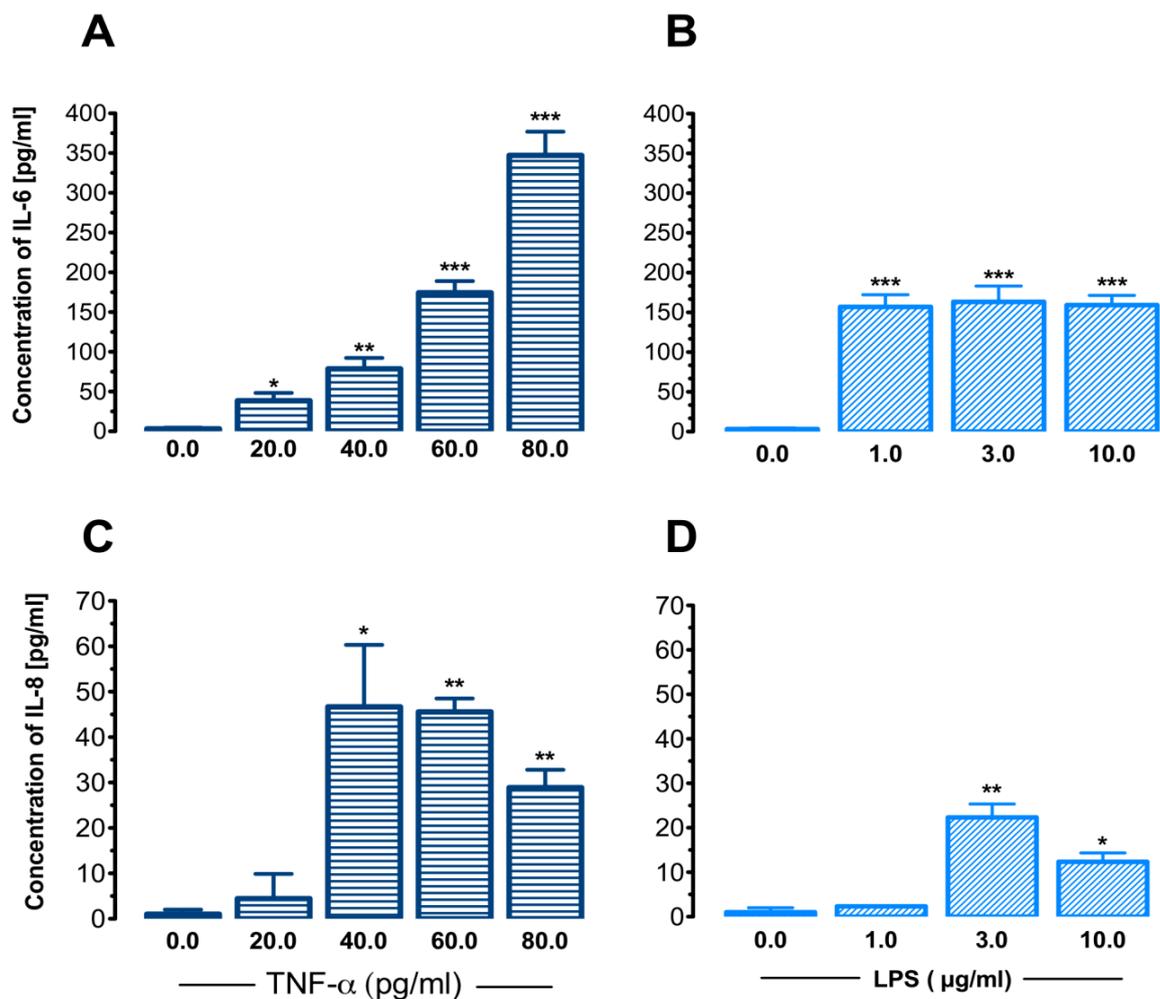
Figure 3.45   Effect of TNF-α and LPS on IL-6 and IL-8 release from extracted bovine chondrocytes.

Bovine chondrocytes were extracted from cartilage explants and cultured for plated for 24 h in serum-free 380 mOsm DMEM prior to stimulation. Panel A and C: TNF-α (20.0-80.0 pg/ml) and Panel B and D: LPS (1.0-3.0 μg/ml) for 6 h. Cell-free supernatants were collected and analysed for IL-6 IL-8 levels by ELISA. Data are presented as Mean ± SEM of n=4 independent experiments performed in triplicate. *p≤ 0.05, **p≤ 0.01 vs. untreated samples (0.0).
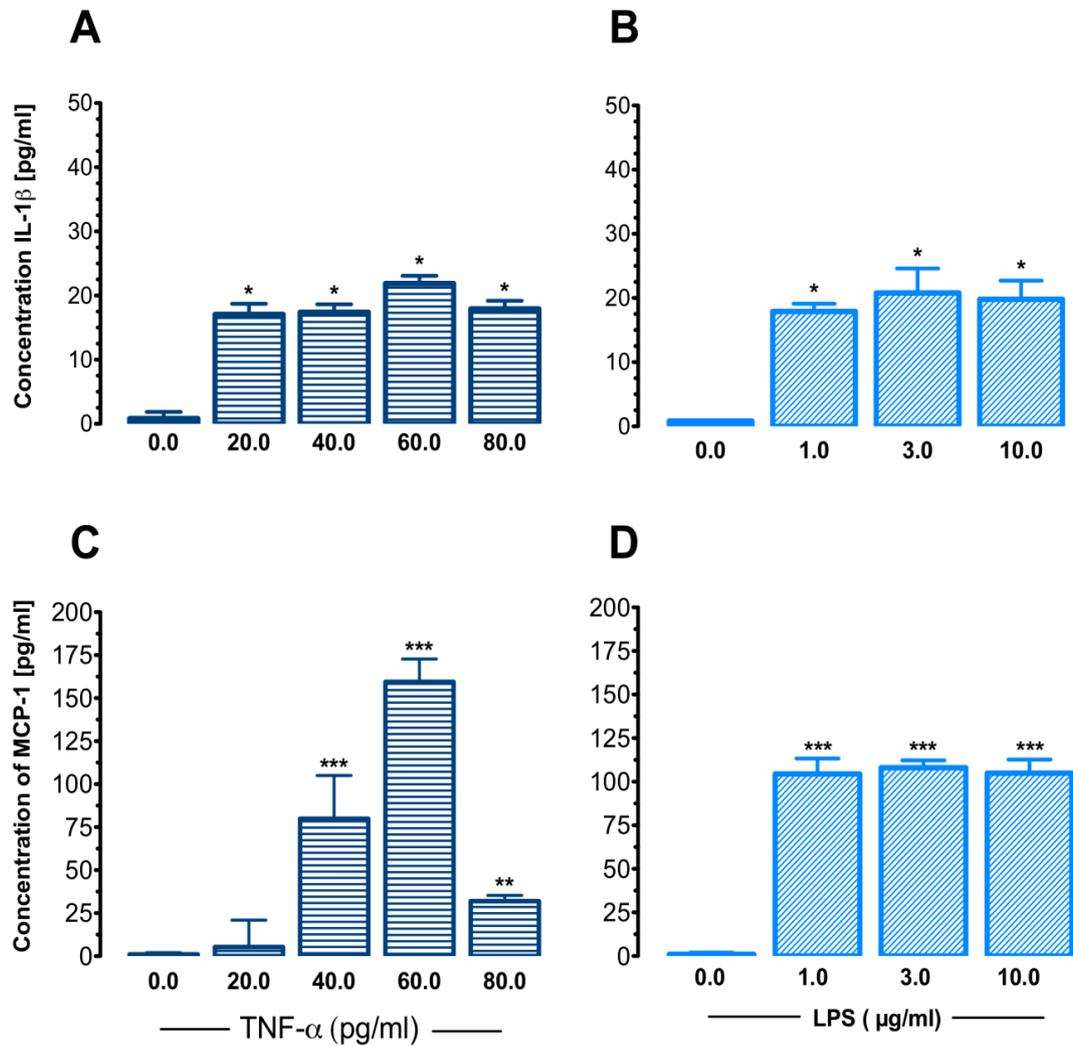
Figure 3.46   Effect of TNF-α and LPS on IL-1β and MCP-1 release from extracted bovine chondrocytes.

Bovine chondrocytes were extracted from cartilage explants and cultured for plated for 24 h in serum-free 380 mOsm DMEM prior to stimulation. Panel A and C: TNF-α (20.0 – 80.0 pg/ml) and Panel B and D: LPS (1.0 – 3.0 μg/ml) for 6 h. Cell-free supernatants were collected and analysed for IL-1β (Panels A, B) and MCP-1 (Panels C, D) levels by ELISA. Data are presented as Mean ± SEM of n=4 independent experiments performed in triplicate. **$p \leq 0.01$, ***$p \leq 0.001$) vs. untreated controls (0.0).

3.4.2 Melanocortin receptors expression in bovine articular chondrocytes.

Following the identification of cytokine release from primary bovine chondrocytes, expression of MC was determined. Gene and species specific primers were designed for both MC_1 and MC_5 in order to determine whether MC_1 and MC_5 were expressed in the articular chondrocytes of *Bos taurus*. However, the full coding sequence of MC_3 has not been revealed yet, therefore it was not possible to investigate its expression at that point. Total mRNA was extracted from non-stimulated chondrocytes extracted from at least 5 individual bovine joints and conventional RT-PCR used to quantify the transcriptional levels of both genes.

There was a marked expression of MC_1 that was consistent between the individual animals and confirmed by the detection of size-specific bands on 2 % agarose gels, corresponding to the expected size of MC_1 fragment of interest (Figure 3.47). The transcriptional studies of MC_5 were non-conclusive, since the expression of the gene varied significantly between the tested animal tissues. All samples were run alongside GAPDH internal control.
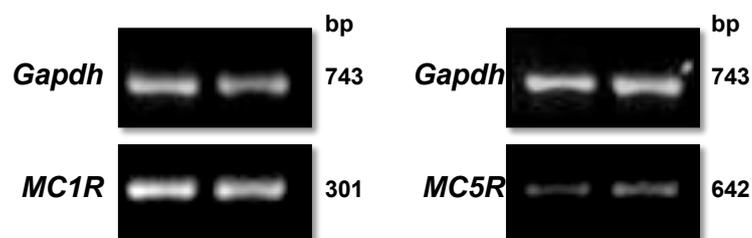
Figure 3.47 Endogenous expression of MC_1 and MC_5 in primary bovine chondrocytes.

Freshly extracted bovine primary chondrocytes were cultured for 24 h in serum-free 380 mOsm DMEM media and total RNA was subsequently extracted. PCR amplification with the respective primers for bovine MC_1 and MC_5 were used to detect and quantify gene expression on 2% agarose gels in triplicates with *Gapdh* used as internal control. Densitometrically quantified MC_1 and MC_5 shown in arbitrary units, each value normalized to the respective GAPDH expression. Data is presented as Mean ± SEM of cartilage from 5 different animals.

3.4.3 Effect of melanocortin peptides on pro-inflammatory cytokine release from TNF-α stimulated primary bovine chondrocytes.

Since the coding region of MC_3 was not available to design the primers needed for the investigative study of MC_3 expression, another approach was used that allowed research into whether MC_3 was expressed by using the selective MC_3 agonist [DTRP[8]]-γ-MSH. The pan-agonist α-MSH, MC_3 agonist D[TRP][8]-γ-MSH and $MC_{3/4}$ antagonist/MC_1 agonist SHU9119 were used in separate experiments to reveal whether MC_1 and MC_3 were present and able to modulate the release of pro-inflammatory mediator release from primary bovine articular chondrocytes.

In order to investigate the validity and efficacy of melanocortin peptides at inhibiting the release of pro-inflammatory cytokines following TNF-α stimulation, they were investigated in primary bovine chondrocytes (Figure 3.48). Primary bovine chondrocytes were extracted from bovine cartilage and monolayer cultures incubated in serum-free 380 mOsm DMEM medium for 24 h prior to pre-treatment for 30 min with α-MSH, D[TRP][8]-γ-MSH or SHU9119 (1.0 – 30.0 μg/ml) prior to stimulation with TNF-α (60.0 pg/ml) for 6 h. Pre-treatment of primary bovine chondrocytes with α-MSH, D[TRP][8]-γ-MSH and SHU9119 caused a significant decrease in IL-β levels. Concentrations between 1.0 and 10.0 μg/ml of α-MSH significantly reduced the levels of IL-1β produced by TNF-α from 21.87 ± 0.23 pg/ml down to 12.85 ± 0.29 pg/ml (α-MSH 1.0 μg/ml), representing a 41% decrease ($p \leq 0.05$). α-MSH (3.0 μg/ml) decreased IL-1β concentration to 13.30 ± 0.71 pg/ml, a 39 % reduction ($p \leq 0.05$). Increasing the peptide concentration did not alter the inhibition of IL-1β compared to the lower 3.0 μg/ml concentration with a similar 39 % reduction. Higher concentrations of α-MSH (30.0 μg/ml) did not have a statistically significant effect (17.34 ± 1.4 pg/ml; $p > 0.05$; Figure 3.48) compared to TNF-α–treated controls.

Treatment of primary chondrocytes with D[TRP][8]-γ-MSH caused a bell shaped concentration dependent decrease in IL-1β levels with 1.0 μg/ml being essentially inactive (19.68 ± 1.58 pg/ml; $p > 0.05$). Levels of IL-1β were reduced by 61 % following stimulation with 3.0 μg/ml ($p \leq 0.05$) and a maximal effect of 76 % at 10.0 μg/ml ($p \leq 0.05$), and starting to slowly decrease efficacy when the

concentration was increased to 30.0 µg/ml (39%). A modest inhibitory effect was observed with SHU9119 with 1.0 µg/ml (16.84±0.72 pg/ml; $p \leq 0.05$) and 3.0 µg/ml (17.46 ± 0.9 pg/ml; $p \leq 0.05$) causing a reduction of 23 % and 20 % respectively. Higher concentrations had no significant effect on TNF-α induced IL-1β levels (Figure 3.48).
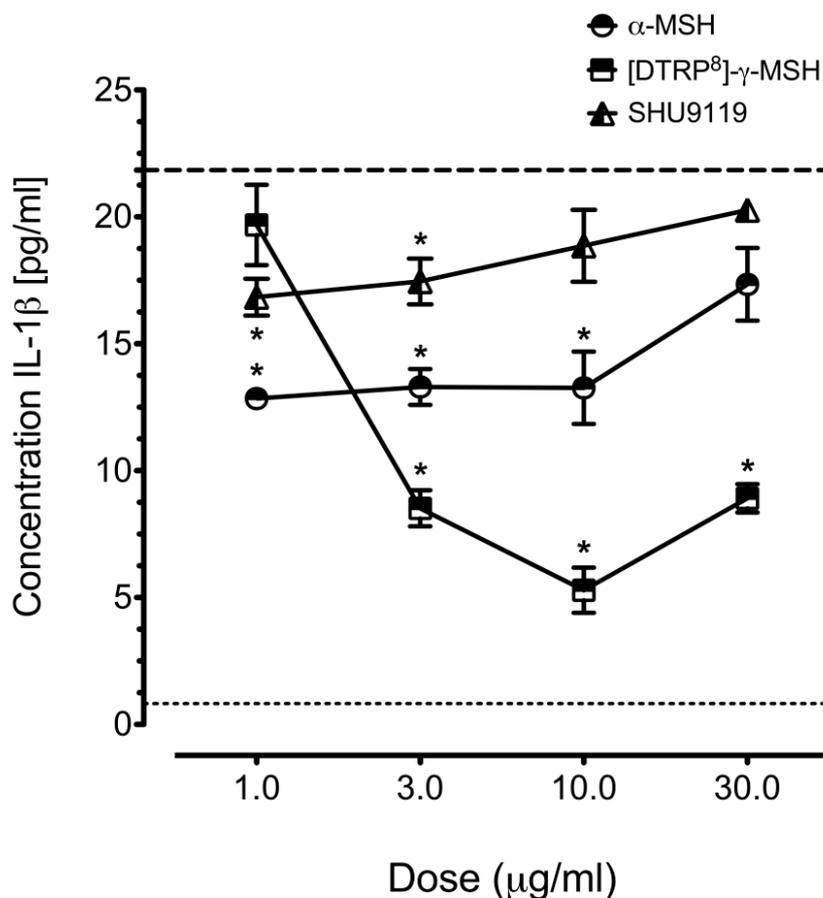
Figure 3.48 Effect of melanocortin peptides on IL-1β release from TNF-α activated primary bovine chondrocytes.

Bovine chondrocytes were extracted from bovine cartilage and cultured for 24 h in serum-free 380 mOsm DMEM medium. Chondrocytes pre-treated with α-MSH, D[TRP][8]-γ-MSH or SHU9119 (1.0-30.0 µg/ml) for 30 mins, prior to stimulation with TNF-α (60.0 pg/ml) for 6 h. Cell-free supernatants were collected and analysed for IL-1β levels by ELISA. Data are presented as Mean± SEM of n=4 independent experiments repeated in triplicate, *$p \leq 0.05$ vs. culture treated with TNF-α alone. Dashed line represents TNF-α treatment; dotted line – untreated cultures.

α-MSH caused a significant abrogation of TNF-α-induced IL-6 release from primary bovine chondrocytes (Figure 3.49 A). TNF-α-stimulation alone caused 174.38 ± 14.6 pg/ml of IL-6 release. α-MSH was most effective at 1.0 μg/ml causing a 92.7 % inhibition in IL-6 release (12.74 ± 1.2 pg/ml; p≤ 0.001). Higher concentrations of peptide still exerted a significant inhibitory effect with 3.0 μg/ml causing an 87.3 % decrease (22.18 ± 0.9 pg/ml; p≤ 0.001), 10.0 μg/ml causing a 76.4 % reduction (41.22 ± 0.57 pg/ml; p≤ 0.001) and 30.0 μg/ml a 48% decrease (90.8 ± 10.24 pg/ml; p≤ 0.01) compared to control.

Following identification of the inhibitory effect of α-MSH, the MC$_3$ agonist D[TRP]^8-γ-MSH was evaluated. D[TRP]^8-γ-MSH (1.0 – 10.0 μg/ml) caused a bell shaped inhibitory effect of IL-6 release (Figure 3.49 A). The maximal effect was observed at 3.0 μg/ml with a 72 % decrease in IL-6 release, whilst 1.0 and 10.0 μg/ml had a reduced inhibitory effect displaying 40.1 % (104.46 ± 3.11 pg/ml; p≤ 0.01) and 48.2 % (90.34 ± 16.29 pg/ml; p≤ 0.01) reduction respectively. Higher concentrations (30.0 μg/ml) did not cause a statistically significant change in the release of IL-6 ($p> 0.05$) compared to TNF-α alone. The MC$_{3/4}$ antagonist/MC$_1$ agonist SHU9119 failed to inhibit IL-6 release at all concentrations evaluated (Figure 3.49 A).

D[TRP]^8-γ-MSH attenuated IL-8 release elicited by TNF-α (60.0 pg/ml) in a bell shaped dependent fashion (Figure 3.49 B). The maximal effect was reached at 3.0 μg/ml causing a 69.4 % decrease in IL-8 levels (13.93 ± 1.13 pg/ml; p≤ 0.01) compared to TNF-α treated controls (45.56 ± 2.94 pg/ml), a similar reduction was observed at 1.0 μg/ml with a 66.5 % decrease. At 10.0 μg/ml a 57 % inhibition was observed, however at 1.0 and 30.0 μg/ml of D[TRP]^8-γ-MSH failed to inhibit IL-8 release ($p> 0.05$). In contrast all concentrations of α-MSH evaluated caused a significant inhibition of IL-8 with a maximal inhibition being observed at 3.0 μg/ml of 66.02 % (15.49±1.28 pg/ml). As previously observed with IL-6, SHU9119 failed to inhibit significantly inhibit IL-8 release at all concentrations evaluated ($p≤ 0.05$; Figure 3.49 B).
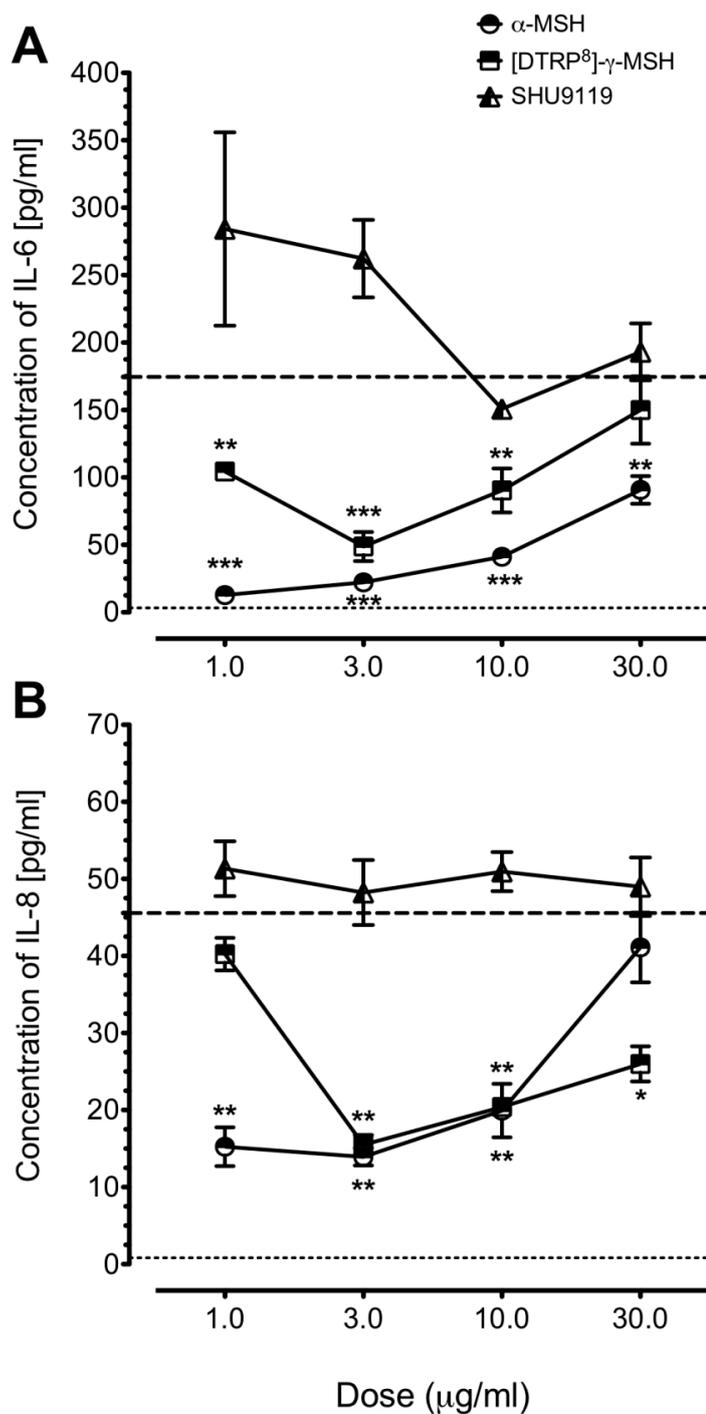
Figure 3.49 Effect of melanocortin peptides on IL-6 and IL-8 release from TNF-α-activated primary bovine chondrocytes.

Bovine chondrocytes were extracted from bovine cartilage and cultured for 24 h in serum-free 380 mOsm DMEM medium. Chondrocytes pre-treated with α-MSH, D[TRP]^8-γ-MSH or SHU9119 (1.0-30.0 μg/ml) for 30 mins, prior to stimulation with TNF-α (60.0 pg/ml) for 6 h. Cell-free supernatants were collected and analysed for IL-6 and IL-8 levels by ELISA. Data is presented as Mean ± SEM of n=4 independent experiments repeated in triplicate, *$p \leq$ 0.05, **$p \leq$ 0.01, ***$p \leq$ 0.001 vs. TNF-α alone.

3.4.4 Effect of MC$_{3/4}$ antagonist SHU9119 on anti-inflammatory effect of α-MSH and D[TRP]^8-γ-MSH in TNF-α activated bovine primary chondrocytes.

To try and unravel whether MC$_1$ and/or MC$_3$ was mediating the effects of the melanocortin peptides α-MSH and D[TRP]^8-γ-MSH, SHU9119 was added on TNF-α activated primary P$_0$ bovine articular chondrocytes. Primary P$_0$ bovine articular chondrocytes were extracted and cultured in a monolayer with serum-free 380 mOsm DMEM medium for 24 h prior to stimulation. The cells were treated with the peptides (1.0 – 10.0 µg/ml) for 30 min ± SHU9119 (10.0 µg/ml) prior to stimulation with TNF-α (60.0 pg/ml) for 6 h and IL-1β, IL-6 and IL-8 levels were detected by ELISA (Figure 3.50). SHU9119 failed to significantly block the effect of α-MSH at 1.0 and 3.0 µg/ml ($p>0.05$), surprisingly however, at 10.0 µg/ml α-MSH the inhibitory effect on IL-1β was blocked in the presence of SHU9119 (Figure 3.50 A). In contrast SHU9119 (10.0 µg/ml) abrogated the anti-inflammatory effect of D[TRP]^8-γ-MSH at all concentrations evaluated (Figure 3.50 B).

α-MSH inhibitory effect on IL-6 was not blocked in the presence of SHU9119 at 1.0 and 3.0 µg/ml. (Figure 3.51 A) However when SHU9119 (10.0 µg/ml) was combined with higher doses of α-MSH (10.0 µg/ml), there was a synergistic effect that caused a marked decrease in IL-6 levels (13.33 ± 3.22 pg/ml) a 66.7 % reduction compared to α-MSH alone (41.22 ± 0.56 pg/ml). In contrast D[TRP]^8-γ-MSH at all concentrations evaluated (1.0 – 10.0 µg/ml) was completely blocked when SHU9119 was given in combination (Figure. 3.51 B). IL-8 released following TNF-α stimulation of P$_0$ chondrocytes showed that SHU9119 (10.0 µg/ml) failed to inhibit the anti-cytokine effects of α-MSH (1.0 – 10.0 µg/ml, Figure 3.51 C). D[TRP]^8-γ-MSH inhibition of IL-8 was abrogated in the presence of SHU9119 (Figure 3.51 D).

Finally, given that SHU9119 blocked the action of D[TRP]^8-γ-MSH but not of α-MSH, we evaluated the effect of this combination on IL-10 synthesis. Treatment of the P$_0$ bovine chondrocytes with α-MSH led to significant increase in IL-10 levels (Figure 3.52 A), there was no synergistic effect when cells were incubated

with a combination of α-MSH and SHU9119 and the peptide's actions were not significantly affected by the addition of the $MC_{3/4}$ antagonist. There was a general reduction in the melanocortin effect, which could be attributed to its partial binding affinities to MC_3 in contribution to MC_1. However, SHU9119 prevented D[TRP]^8-γ-MSH from binding to its receptor, therefore reduced significantly IL-10 production (Figure 3.52 B). SHU9119 caused 89 % reduction of IL-10 in 1.0 µg/ml and 10.0 µg/ml of D[TRP]^8-γ-MSH treated cells, and 86 % when given simultaneously with 3.0 µg/ml of D[TRP]^8-γ-MSH.
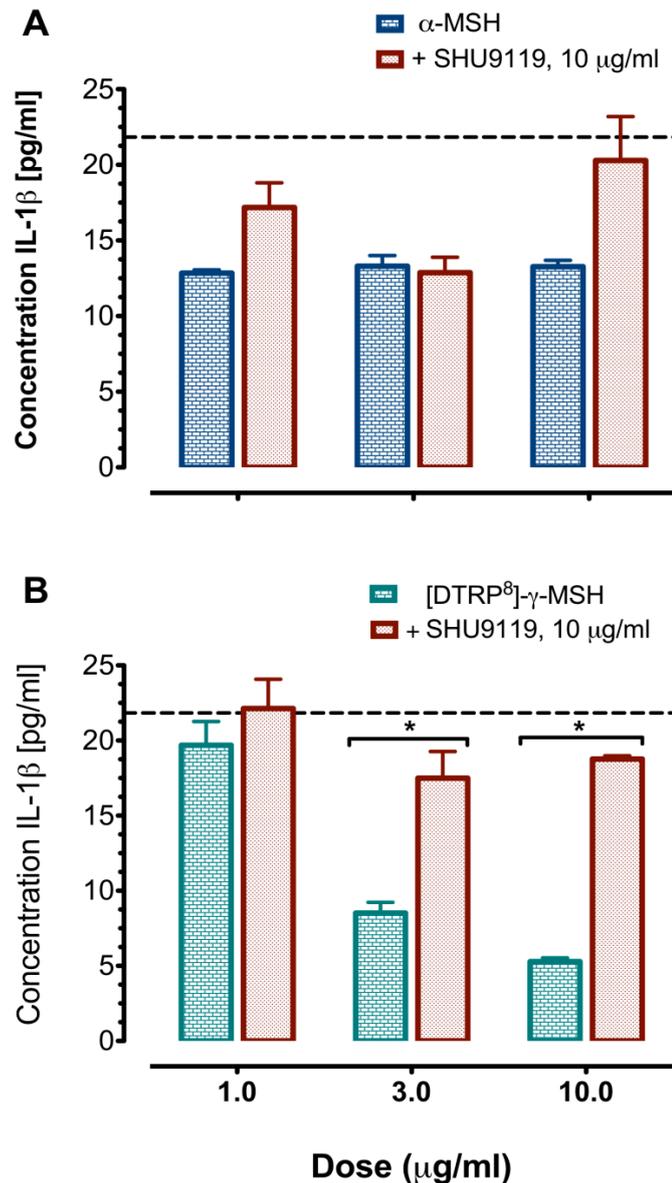
Figure 3.50 Effect of the MC$_{3/4}$ antagonist SHU9119 on IL-1β release from TNF-α activated primary bovine articular chondrocytes following α-MSH and D[TRP]^8-γ-MSH treatment.

Bovine chondrocytes were extracted from bovine cartilage and cultured for 24 h in serum-free 380 mOsm DMEM medium. Chondrocytes were then pre-treated with α-MSH (Panel A) or D[TRP]^8-γ-MSH (1.0 – 10.0 μg/ml; Panel B) ± SHU9119 (10.0 μg/ml) for 30 mins, prior to stimulation with TNF-α (60.0 pg/ml) for 6 h. Cell-free supernatants were collected and analysed for IL-1β levels by ELISA. Data are presented as Mean ± SEM of n=4 independent experiments repeated in triplicate, *$p \leq$ 0.05, **$p \leq$ 0.01 vs. TNF-α alone.

Figure 3.51. Effect of the MC$_{3/4}$ antagonist SHU9119 on IL-6 release from TNF-α activated primary bovine articular chondrocytes following α-MSH and D[TRP]^8-γ-MSH treatment.

Bovine chondrocytes were extracted from bovine cartilage and cultured for 24 h in serum-free 380 mOsm DMEM medium. Chondrocytes were then pre-treated with α-MSH (Panel A) or D[TRP]^8-γ-MSH (Panel B; 1.0 – 10.0 μg/ml) ± SHU9119 (10.0 μg/ml) for 30 mins, prior to stimulation with TNF-α (60.0 pg/ml) for 6 h. Cell-free supernatants were collected and analysed for IL-6 levels by ELISA. Data is presented as Mean ± SEM of n=4 independent experiments repeated in triplicate, *p≤ 0.05, **p≤ 0.01 vs. TNF-α alone.
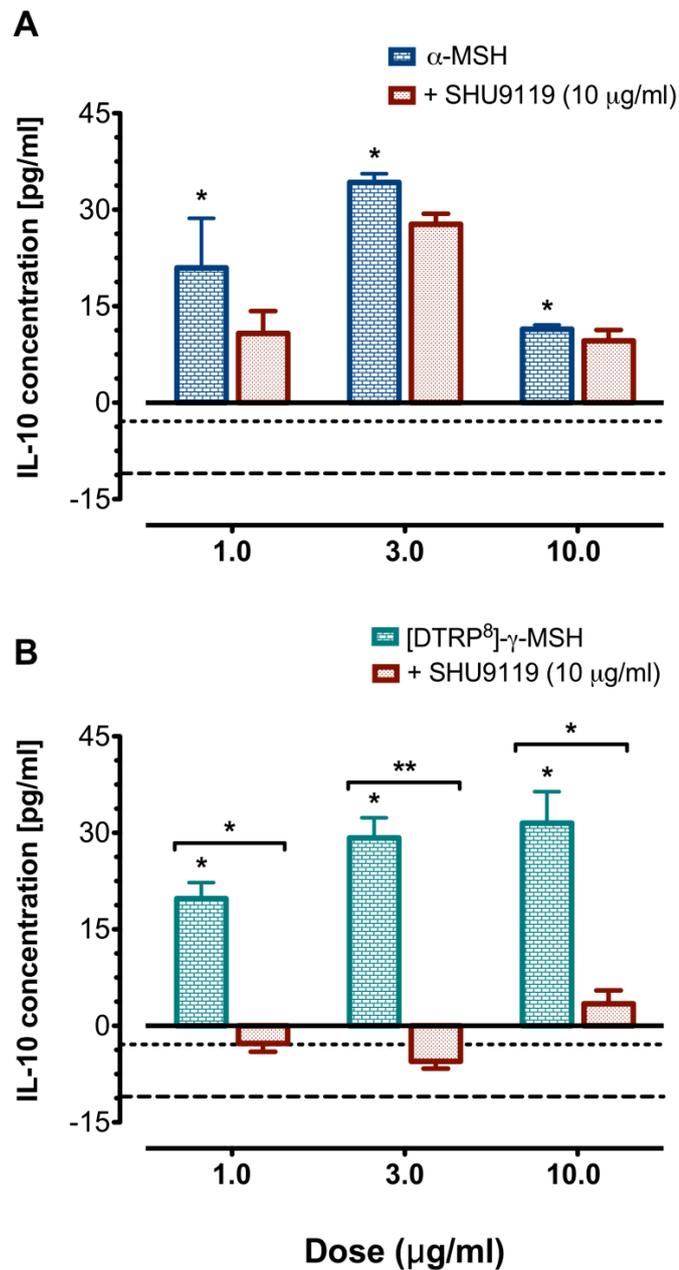
Figure 3.52 Effect of the MC$_{3/4}$ antagonist SHU9119 on IL-10 release from TNF-α activated primary bovine articular chondrocytes following α-MSH and D[TRP]^8-γ-MSH treatment.

Bovine chondrocytes were extracted from bovine cartilage and cultured for 24 h in serum-free 380 mOsm DMEM medium. Chondrocytes were then pre-treated with α-MSH (Panel A) or D[TRP]^8-γ-MSH (Panel B; 1.0 – 10.0 μg/ml) ± SHU9119 (10.0 μg/ml) for 30 mins, prior to stimulation with TNF-α (60.0 pg/ml) for 6 h. Cell-free supernatants were collected and analysed for IL-10 levels by ELISA. Data is presented as Mean ± SEM of n=4 independent experiments repeated in triplicate, *$p \le 0.05$, **$p \le 0.01$ vs. TNF-α alone.

3.4.5 Cartilage impact and the effect of melanocortins and steroids on bovine chondrocytes *in situ*.

Mechanical stress above the physiological range can profoundly influence articular cartilage causing matrix damage, changes to chondrocyte metabolism and cell injury/death (Bush and Hall, 2005) and therefore has been implicated as a risk factor for the development of osteoarthritis. The mechanism of cell damage is not understood, but chondrocyte volume could be a determinant of the sensitivity and subsequent response to load (Hall, 1998). In OA, it is possible that the chondrocyte swelling that occurs renders the cells more sensitive to the damaging effects of mechanical stress. Here, changes in viability of *in situ* chondrocytes following a single blunt impact have been investigated in the presence of α-MSH, D[TRP]^8-γ-MSH, and the steroid dexamethasone evaluated at the time of impact and 6h post-impact.

3.4.5.1 Cell viability of articular bovine chondrocytes *in vivo* following single impact.

Explants of bovine articular cartilage were incubated with the fluorescent indicators 5.0 μM Calcein-AM and 1.0 μM Propidium Iodide, permitting the measurement of cell viability, respectively, using confocal laser scanning microscopy (CLSM). Cartilage was then subjected to a single impact with 1.14 N force delivered from a vertical drop tower, which caused areas of chondrocyte injury/death within the superficial zone (SZ) as shown on Figure 3.52.

Cell death following single impact was quantified using Imaris 7.1.1 Spots analysis software and the results are shown in Figure 3.58. Impact caused a significant (*p<0.05) increase in cell death. Non-impacted cartilage showed 2.95 ± 0.26 % chondrocytes death, which following impact increased to 13.50 ± 1.72% (Figure 3.53). Areas of chondrocyte injury inflicted from impact tower injury can be seen on Figure 3.58 and visually compared to non-impacted counterparts (original 3D volume image reconstitutions [A and B] and spots analysis [a and b] respectively).
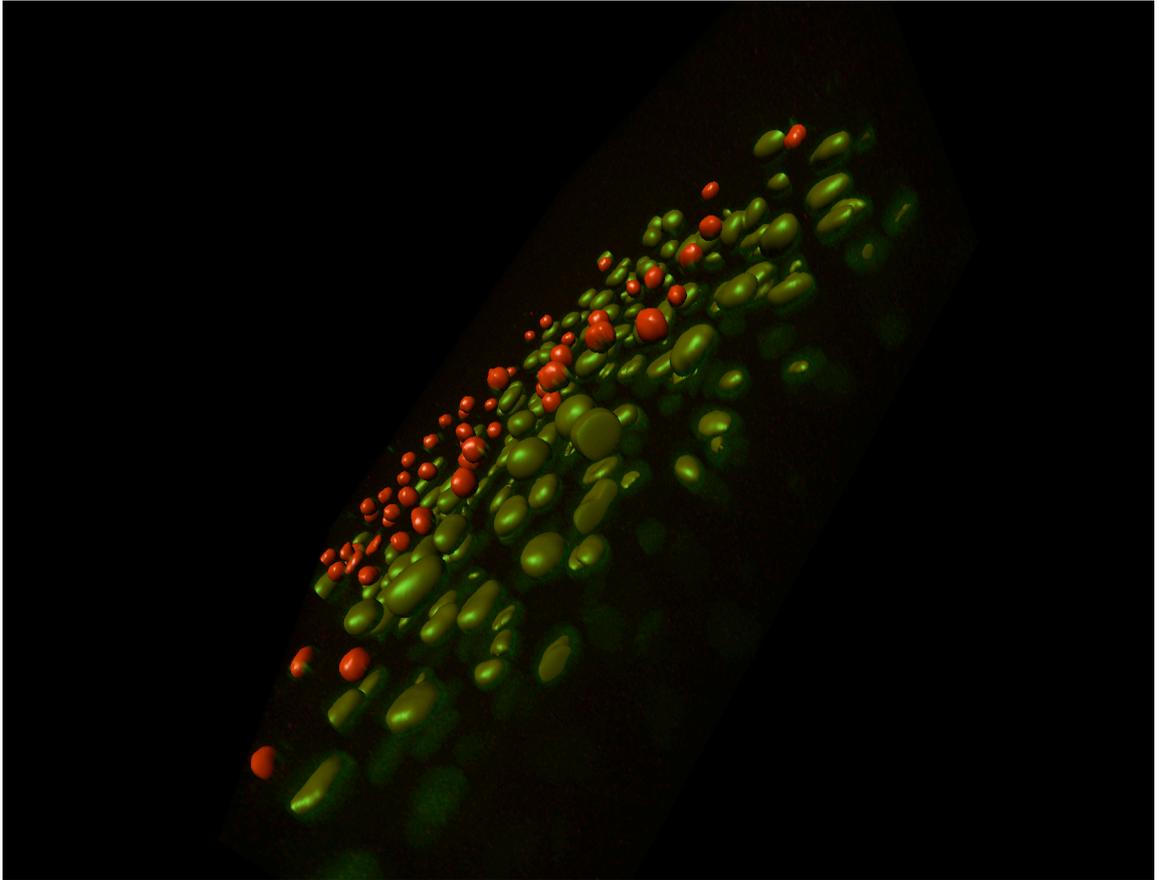
Figure. 3.52. A 3D-image of cartilage impacted chondrocytes.

Cartilage explant was incubated for 30 min at 37ºC with 5.0 μM Calcein-AM and 1.0 μM Propidium Iodine for staining alive and dead cells respectively and visualized using CLSM at x630 magnification using x63 immersable lens. Iso-surface object were created for all cells, in order to create presentation image of an impacted piece of cartilage (image not used for volume analysis) Red cells represent chondrocytes undergoing cell death, and green – cells that are alive and fully functional at the time of imaging.
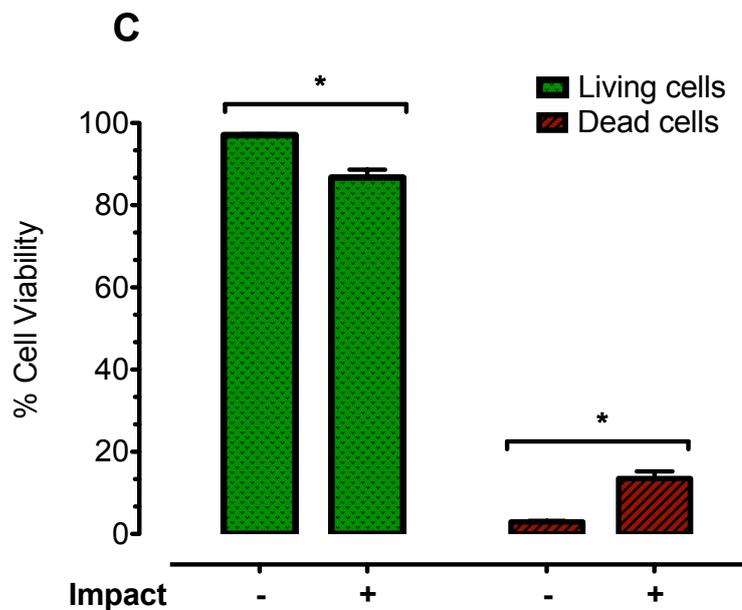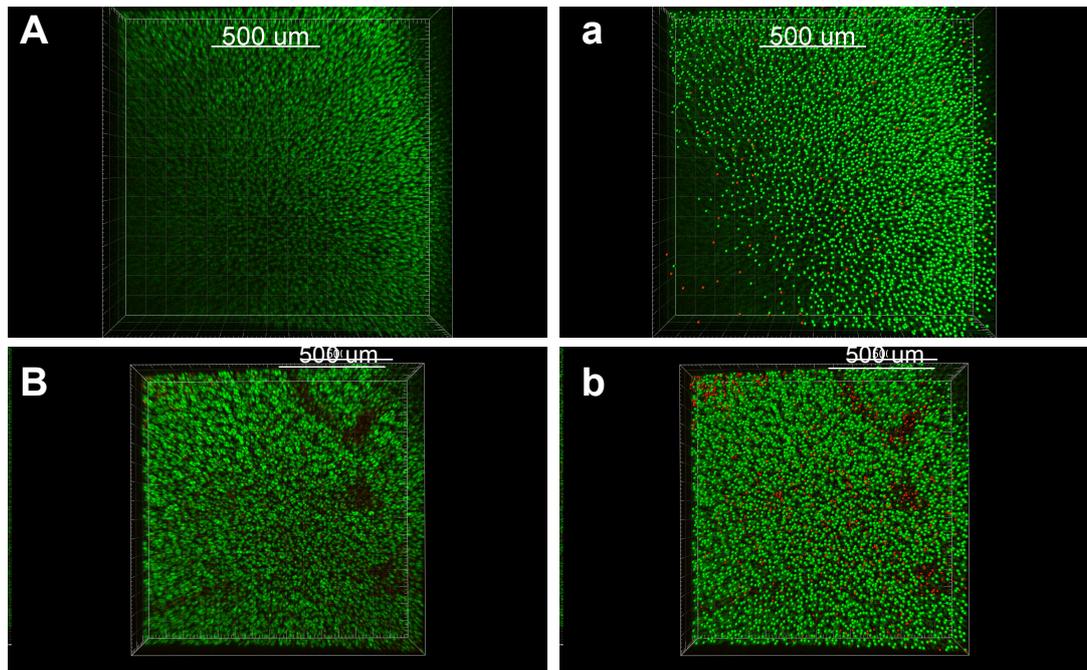
Figure 3.53 Effect of single impact on cell death as quantified by Imaris 7.1.1 Spots Software.

Bovine articulate cartilage explants were incubated with the fluorescent indicators Calcein-AM (5.0 μM) and Propidium Iodide (1.0 μM) prior to (Panel A, a) and at 6 h post-mechanical trauma (Panel B, b) for 30 min, and visualized under CLSM. Imaris 7.1.1 Spot Analysis software was used to quantify the number of viable (green) and non-viable (red) cells. Panels a, and b represent Spots analysis of the original CLSM images, taken at x100 magnification (Panels A and B). Percentage cell death was calculated based on the acquired results (Panel C). Data are representative images or Mean ± SEM of n=4 experiments, repeated in triplicates, *$p \le$ 0.05 vs. non-impacted control samples.

3.4.5.1.1 The effect of melanocortins and dexamethasone on cell death.

In order to investigate whether the melanocortin peptides have chondroprotective properties, cell viability was quantified as described earlier. Briefly, cells were treated for 6 h with 3.0 μg/ml α-MSH or D[TRP]^8-γ-MSH, or 1.0 μM dexamethasone and then incubated with the fluorescent indicators Calcein-AM (5.0 μM) and Propidium Iodide (1.0 μM) for 30 min prior to CLSM, permitting the measurement of cell viability.

Figure 3.54 demonstrates that α-MSH caused a reduction in chondrocyte death compared to basal levels (2.95 %). Cell death decreased to 0.97 %, when cartilage explants were incubated for 6 h with α-MSH (3.0 μg/ml) prior to CLSM imaging, representing 67 % reduction in cell death, compared to untreated controls ($p\le$ 0.05). However, neither D[TRP]^8-γ-MSH (3.0 μg/ml) nor dexamethasone (1.0 μM) was able to show any significant effect on this parameter.
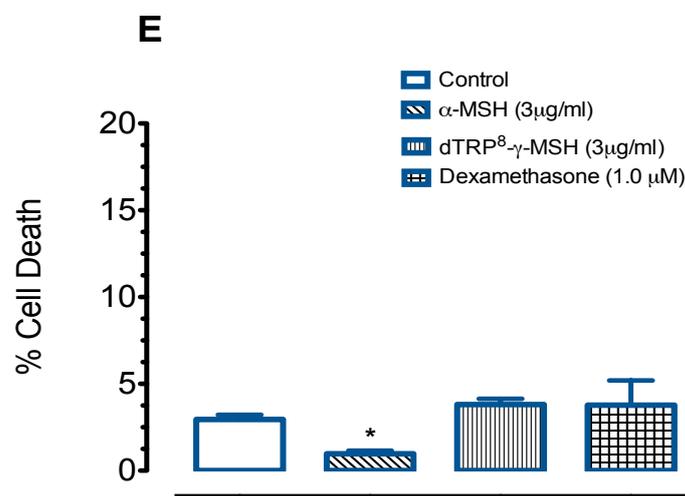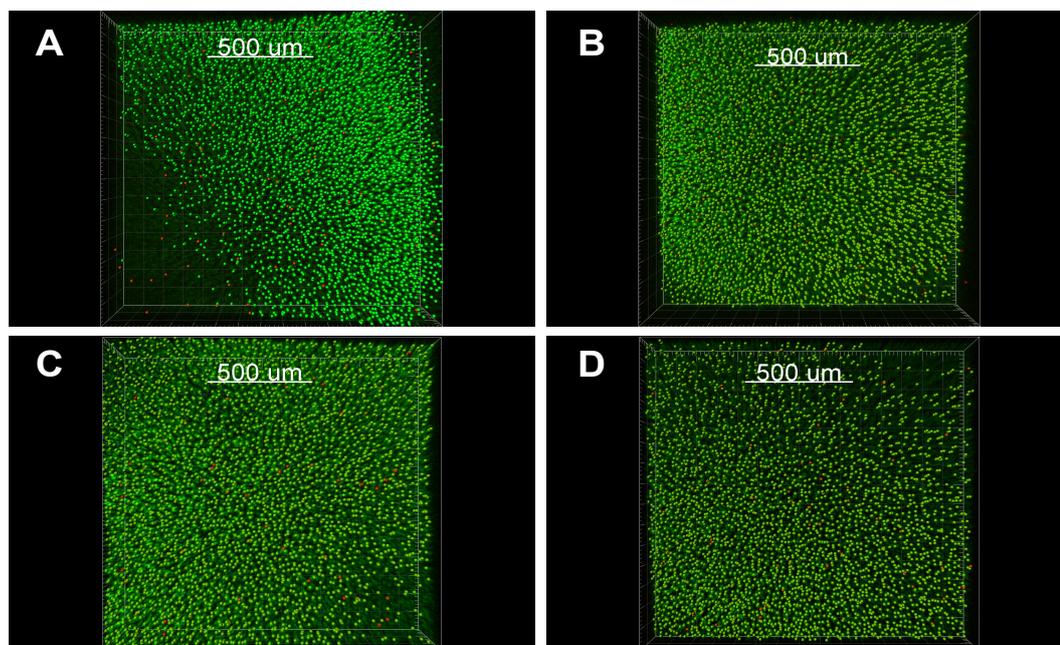
Figure 3.54. Chondroprotective effect of melanocortin peptides as quantified by Imaris 7.1.1 Spots Analysis software.

Bovine articular cartilage explants were treated with either DMEM (Panel A), α-MSH (3.0 μg/ml; Panel B), D[TRP]^8-γ-MSH (Panel C), or 1.0 μM dexamethasone (Panel D) for 6 h. Cartilage explants were then incubated for 30 min at 37°C with the fluorescent indicators Calcein-AM (5.0 μM) and Propidium Iodine (1.0 μM) for staining alive and dead cells respectively, immediately following which the explants were visualized using CLSM at x100 magnification. Imaris 7.1.1 Spot Analysis software was used to quantify the number of viable (green) and non-viable (red) cells. Percentage cell death was calculated based on the acquired results (Panel E). Data are representative images or Mean ± SEM of n=4 experiments, repeated in triplicates, *$p \leq$ 0.05 vs. non-impacted control samples.

3.4.5.1.2 The effect of melanocortins and dexamethasone on pro-inflammatory cytokine release from non-impacted bovine chondrocytes *in situ*.

In order to determine the basal levels of pro-inflammatory cytokines released from non-impacted bovine cartilage explants, the cartilage pieces were incubated in 380 mOsm DMEM for 24 h, following which the medium was replaced with fresh DMEM and IL-1β, IL-6 and IL-8 release was detected at 6 h by ELISA. Similarly, the effect of the melanocortins on pro-inflammatory cytokine release from non-injured cartilage pieces were evaluated in the supernatants at 6 h, post-treatment of the bovine explants with α-MSH (3.0 µg/ml), D[TRP]^8-γ-MSH (3.0 µg/ml) or dexamethasone (1.0 µM) and compared to the basal levels of cytokine release (Figure 3.55).

There was a significant basal production of cytokines detected in the supernatants of non-treated bovine articular cartilage explants, with 17.94 ± 4.61 pg/ml/g (pg/ml per gram of cartilage tested) of IL-1β, 59.33 ± 8.75 pg/ml/g of IL-6 and 97.11 ± 10.57 pg/ml of IL-8, values which were not significantly altered by the addition of any of the drugs as can be seen from Figure 3.55 Panels A, B and C respectively.
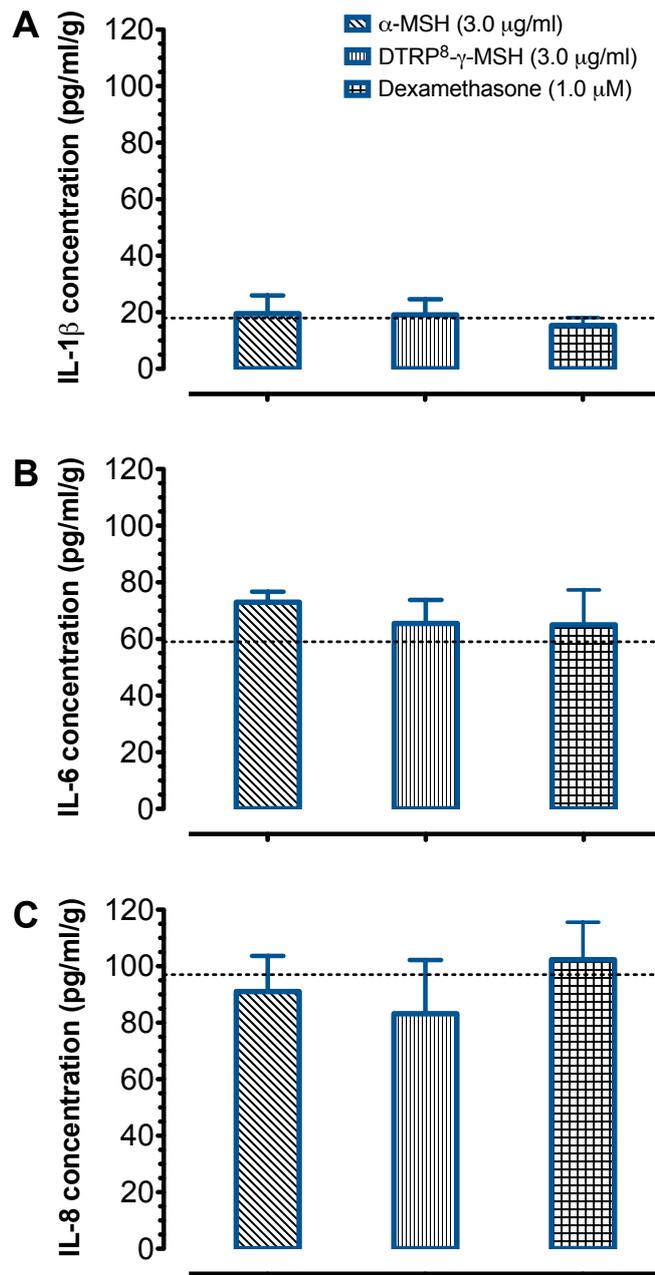
Figure 3.55 Effect of α-MSH, D[TRP]^8-γ-MSH and dexamethasone on basal production of IL-1β, IL-6 and IL-8.

Bovine articular cartilage explants were treated with DMEM, α-MSH (3.0 μg/ml), [DTRP]^8-γ-MSH (3.0 μg/ml), or dexamethasone (1.0 μM) for 6 h and cell-free supernatants were collected and analysed for IL-1β (Panel A), IL-6 (Panel B) and IL-8 (Panel C) via commercially available ELISAs (R&D Systems). Results were corrected per gram of tissue tested. Data are presented as Mean ± SEM of n=4 experiments repeated in triplicate. DMEM-treated control samples are represented by a dotted line.

3.4.5.2 The effect of melanocortin peptides and dexamethasone on impacted cartilage explants.

3.4.5.2.1 The effect of melanocortin peptides and dexamethasone on cell death as detected via CLSM.

In order to investigate whether melanocortin peptides were able to exhibit a protective effect on impacted bovine chondrocytes *in situ*, cell viability was quantified as described previously. Briefly, cells were treated for 30 min with DMEM, α-MSH (3.0 μg/ml), D[TRP]^8-γ-MSH (3.0 μg/ml), or dexamethasone (1.0 µM) prior to impact and incubated for 6 h. 30 min prior to CLSM imaging, cartilage explants were incubated with the fluorescent indicators Calcein-AM (5.0 µM) and Propidium iodide (1.0 µM).

All drugs caused a significant decrease in cell death ($p \leq 0.05$) compared to DMEM-treated impacted sample (Figure 3.56). Upon single blunt impact using the vertical drop tower, 13.5 ± 1.72 % of the cells in the injured cartilage were found to be dead 6 h following the impact. Treatment of cartilage explants with melanocortin peptides or dexamethasone for 30 min prior to impact and left to incubate for 6 h resulting in significant protective effects on the chondrocytes. Cell death was reduced to 5.10 ± 1.42 %, when the articular cartilage explants were incubated with α-MSH (3.0 µg/ml), representing a 62% reduction ($p \leq 0.05$). Similar was the effect of the selective MC$_3$ agonist D[TRP]^8-γ-MSH with the peptide protecting 66 % of the cells during the first 6 h of impact, allowing for only 4.63 ± 0.62 % of the chondrocytes to undergo cell death ($p \leq 0.05$). Similarly, dexamethasone exhibited chondroprotective properties and caused a 50 % reduction in cell death compared to DMEM-treated impacted cartilage explant with just 6.75 ± 2.38 % of the cells in the injured explant to undergo cell death ($p \leq 0.05$).

3.4.5.2.2 Melanocortin peptides and dexamethasone decrease pro-inflammatory cytokines induced by mechanical trauma.

In order to investigate the involvement of pro-inflammatory cytokines in the chondrocyte function following cartilage injury, bovine articular cartilage explants were subjected to a single impact with a force of 1.12 N. Following the

mechanical trauma, the cartilage were returned to their media for 6 h, when cell-free supernatants were collected. Similarly, the cartilage explants were treated with the melanocortin peptides and dexamethasone for 30 min, following which period they were subjected to single impact and returned back in the same media containing the respective drug for additional 6 h. The supernatants were then collected and analysed for IL-1β, IL-6 and IL-8 production by ELISA.

Upon impact, the chondrocytes in the DMEM-treated impacted explants produced 351.22 ± 24.12 pg/ml/g IL-1β (Figure 3.57), representing a 19-fold increase ($p \leq$ 0.001) in the release of this pro-inflammatory cytokine compared to the basal amounts released by the chondrocytes from non-impacted cartilage explants. The injury led to 13-fold increase ($p \leq$ 0.001) in IL-6 production compared to basal levels, with 448.41 ± 19.65 pg/ml/g at 6 h post-impact (Figure 3.57 B). Similarly, there was a significant, 3-fold increase in the levels of IL-8 ($p \leq$ 0.001), with 293.46 ± 13.94 pg/ml/g (Figure 3.57 C). All of the drugs were found to be effectively reducing these amounts of pro-inflammatory cytokines, produced by the chondrocytes in response to the mechanical trauma. α-MSH (3.0 µg/ml) decreased the release of IL-1β by 41 % to 209.14 ± 2.22 pg/ml/g ($p \leq$ 0.01) compared to the respective DMEM-treated samples (Figure 3.57 A). The selective MC$_3$ agonist was significantly more potent in inhibiting the release of IL-1β to 175.09 ± 19.13 pg/ml/g (50 %, $p \leq$ 0.01). In similar fashion dexamethasone caused an even larger reduction of 58% of IL-1β release (149.80 ± 2.83 pg/ml/g; $p \leq$ 0.001). IL-6 release was significantly inhibited in the presence of α-MSH, D[TRP][8]-γ-MSH and Dexamethasone down to 158.32 ± 12.87 pg/ml/g, 130.88 ± 7.75 pg/ml/g and 73.57 ± 6.26 pg/ml/g for each of the peptides respectively, therefore representing 65%, 71% and 84% reduction in the secretion of this cytokine ($p \leq$ 0.01). A similar effect on IL-8 release was observed, with α-MSH causing a significant 53 % reduction, with detected levels of 137.26 ± 15.60 pg/ml/g of IL-8 ($p \leq$ 0.01, compared to DMEM-treated impacted controls). D[TRP][8]-γ-MSH led to 54 % decrease down to 133.81 ± 22.05 ($p \leq$ 0.01). Dexamethasone was slightly more effective in reducing IL-8 release with a 65 % decrease (104.10 ± 12.39 pg/ml/g) in the levels, detected in the supernatants of impacted cartilage explant incubated only with DMEM media.
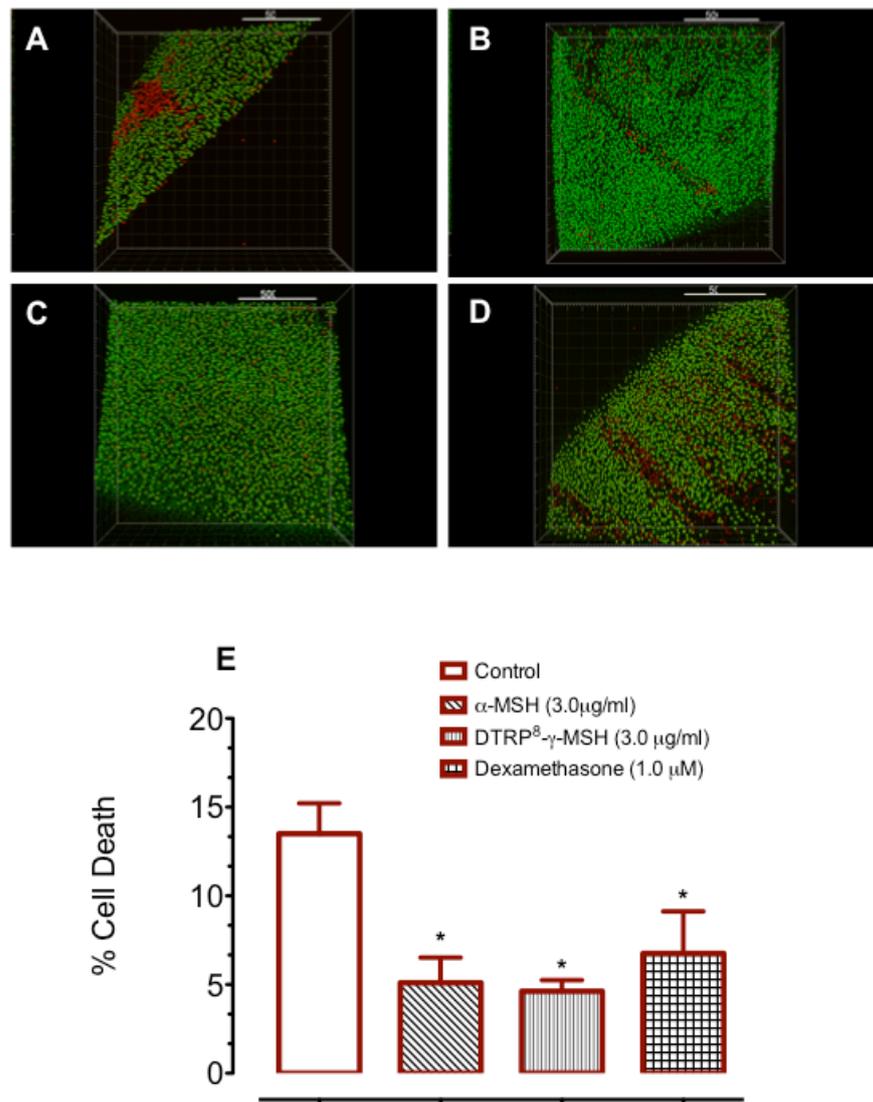
Figure 3.56 Effect of melanocortins and dexamethasone on chondrocyte death following impact as quantified by Imaris 7.1.1 Spots Analysis software.

Bovine articular cartilage explants were treated with either DMEM (Panel A), α-MSH (3.0 μg/ml; Panel B), [DTRP]^8-γ-MSH (3.0 μg/ml Panel C), or 1.0 μM Dexamethasone (Panel D) for 30 min prior to blunt single impact delivered by a vertical drop tower and incubated for 6 h. Cartilage explants were then incubated for 30 min at 37ºC with the fluorescent indicators Calcein-AM (5.0 μM) and Propidium Iodine (1.0 μM) for staining alive and dead cells respectively, immediately following which the explants were visualised using CLSM at x100 magnification. Imaris 7.1.1 Spot Analysis software was used to quantify the number of viable (green) and non-viable (red) cells. Cell death was calculated based on the acquired results (Panel E). Data are representative images or Mean ± SEM of n=4 experiments, repeated in triplicates, *p≤ 0.05 vs. DMEM-incubated impacted controls.
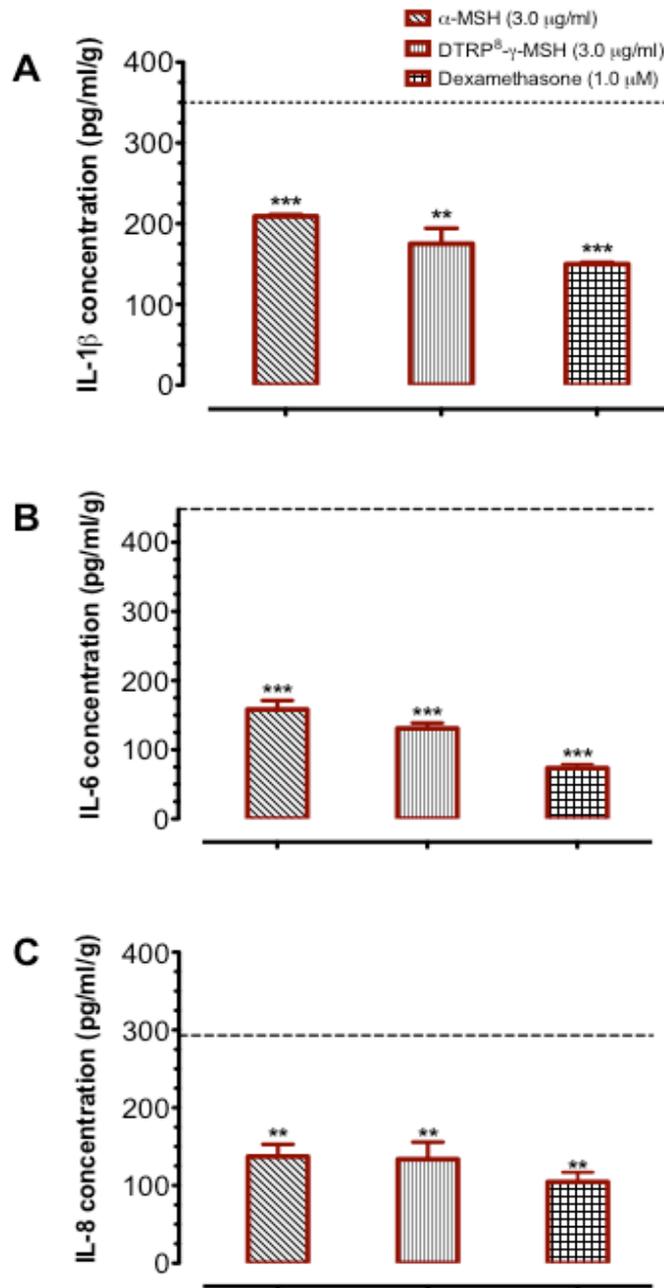
Figure 3.57. Effect of α-MSH, D[TRP]^8-γ-MSH and dexamethasone on basal production of IL-1β, IL-6 and IL-8.

Bovine articular cartilage explants were treated with DMEM, α-MSH (3.0 μg/ml), D[TRP]^8-γ-MSH (3.0 μg/ml), or dexamethasone (1.0 μM) for 6 h and cell-free supernatants were collected and analysed for IL-1β (Panel A), IL-6 (Panel B) and IL-8 (Panel C) via commercially available ELISAs (R&D Systems). Results were corrected per gram of tissue tested. Data are presented as Mean ± SEM of n=4 experiments repeated in triplicate. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to DMEM-treated controls (Dotted line).

Chapter 4
Discussion

Osteoarthritis is a degenerative joint disorder, characterized by inflammation and cartilage degradation (Pelletier *et al.*, 1991, Shinmei *et al.*, 1991, Lotz *et al.*, 1992, Reboul *et al.*, 1996, Melchiorri *et al.*, 1998, Shlopov *et al.*, 2000, Fernandes *et al.*, 2002, Schuerwegh *et al.*, 2003, Goldring *et al.*, 2008, Rai *et al.*, 2008). It has long been known that the body produces endogenous compounds capable of repressing or resolving inflammation. These include annexin-1 (Flower and Blackwell, 1979, Hannon *et al.*, 2003), galectins (La *et al.*, 2003) and in the context of this thesis, the melanocortin peptides (Star *et al.,* 1995, Ichiyama *et al.,* 1999, Getting *et al.,* 2001, 2002, Scholzen *et al.*, 2003). They have been shown to possess a multitude of actions including inhibition of cytokines, chemokines and NO release from MØ (Star *et al.,* 1995, Getting *et al.,* 1999, 2001 Kalden *et al.,* 1999) and reduction of adhesion molecule expression on the endothelium (Kalden *et al.*, 1999, Scholzen *et al.*, 2003), all these actions help to modulate the host's inflammatory response. Given the wealth of knowledge generated so far, few studies have looked at the potential anti-inflammatory effect of melanocortin peptides in osteoarthritis.

This thesis investigates the ability of melanocortins to modulate pro-inflammatory cytokines production (such as IL-1β, IL-6, IL-8, MCP-1) and induce anti-inflammatory protein release (HO-1 and IL-10), but also apoptotic pathways induction and cell death from cell line and primary bovine chondrocytes *in vitro* and *in situ*. This work has allowed for better understanding of their potential role of melanocortin peptides in modulating some of the inflammatory pathways and cell death aspects of degenerative pathologies such as osteoarthritis, but also to ascertain the role of chondrocytes in this disorder.

Several main conclusions can be drawn from the results presented in this thesis. The first converges on the use of an *in vitro* cell culture-based model of chondrocyte inflammation that allows studying the effects of inflammatory mediators on chondrocyte metabolism and cytokine and chemokine production. It has been shown that monolayer cultures, yielding high cell density are one of the best tools for studying the cellular and molecular mechanisms of activated chondrocytes (Finger *et al.*, 2004, Goldring, 2004).

Furthermore, this thesis has evaluated whether the human C-20/A4 chondrocytes are a suitable surrogate model for evaluating the effect of anti-inflammatory drugs including melanocortin peptides on inflammatory mediator release from stimulated cells. The human C-20/A4 chondrocytic cell-line, and primary chondrocytes *in vitro* and *in situ* (cartilage explants) were investigated for expression of pro-inflammatory cytokines and cartilage degrading MMP production upon activation and the role that these cytokines play in cartilage metabolism (collagen type I and type II production) was also studied. These results suggested a possible role for chondrocytes in cartilage inflammation and damage.

Secondly, functionally active MC receptors were detected in human C-20/A4 chondrocytes and primary bovine articular chondrocytes, which upon activation led to a significant induction of cAMP formation, partial repression of pro-inflammatory cytokine and matrix metalloproteinases production, and marked increase in production of the anti-inflammatory proteins HO-1 and IL-10.

Thirdly, it was concluded that inhibition of MC_3 activity by SHU9119 exacerbates the inflammatory response of the chondrocytes and abolishes the potent anti-inflammatory effects displayed by D[TRP]^8-γ-MSH, therefore suggesting a role for this receptor in modulating some of the inflammatory pathways elicited by activation of C-20/A4 chondrocytes.

Finally, the protective effects of melanocortins were evaluated on TNF-α-induced chondrocyte death and inflammation, as well as identification of their cytoprotective and anti-inflammatory role in mechanically injured cartilage explants. During osteoarthritis ECM matrix degradation leads to tissue hydration that signifies a decrease in extracellular osmolarity and leads to chondrocyte sensitization to external stimuli. An *in vitro* model (using C-20/A4 chondrocytes) was established mimicking these conditions, which led to the production of pro-inflammatory cytokines and MMPs and subsequently, the anti-inflammatory properties of the melanocortins in this model were confirmed. In all cases a strong correlation occurred with: melanocortin peptide dampening the pro-inflammatory cytokine synthesis/release, inducing anti-inflammatory proteins and cytokines to aid in the resolution of inflammation and significantly diminishing

chondrocyte cell death resulting from both mechanical trauma (in the case of cartilage explants) and the cytotoxic effect of exogenous TNF-α.

Chondrocytes play an important role in maintaining the integrity of cartillage, during osteoarthritis they become activated leading to the release of an array of pro-inflammatory mediators, apoptosis and alterations in the synthesis of ECM (Pelletier *et al.*, 1991, Goldring *et al.*, 1994b, Shlopov *et al.*, 1997, 2000, Goldring *et al.*, 2011). Immortalised chondrocytic cell lines have been developed to serve as easily accessible, reproducible models of chondrocyte function. Given the difficulty in obtaining human tissue, the use of human cell lines can be invaluable, in trying to dissect the inflammatory pathways involved in chondrocye function and identification of novel treatments for modulating these pathways. Primary cultures of articular chondrocytes isolated from various animal and human sources have served as useful models for studying the mechanisms controlling the response of chondrocytes to various pro-inflammatory and catabolic factors and cytokines (Goldring, 2004). The use of human chondrocytes is problematic and the restricted access to human tissue posses great difficulties for using these cells as a surrogate model. The immortalized chondrocyte cell lines, instead of substituting for primary chondrocytes, may serve as models for increasing knowledge on chondrocyte function not achievable by the use of primary chondrocytes (Finger *et al.*, 2004). Therefore a reproducible source of chondrocytes with human origin would be the most desirable model for studying cartilage function relative to human osteoarthritis (Goldring, 2004) and that is why the human cell line C-20/A4 chondrocytes were used to evaluate chondrocyte's function and response to inflammatory mediators.

Effect of pro-inflammatory stimuli on nitric oxide release and on chondrocyte death.

Inducible nitric oxide synthase (iNOS) gene expresses a calcium calmudolin-independent enzyme which can catalyse NO production from L-arginine. The induction of iNOS activity has been demonstrated in a wide variety of cell types under stimulation with cytokines and LPS. Recent studies have indicated that human articular chondrocytes express iNOS (Schmidt *et al.*, 2010). C-20/A4 chondrocytes monolayer cultures were stimulated with varying concentrations of H_2O_2, TNF-α, LPS and MSU for 6 h and the ability of these stimuli to activate iNOS to produce NO was tested in conjunction with testing their effect on chondrocyte viability. Substantial amounts of NO, released from C-20/A4 cells following stimulation with H_2O_2 were detected at 6 h post stimulation via the Griess assay, demonstrating that the chondrocytes can react to oxidative stress by activating iNOS (Mendes *et al.*, 2003a,b). In particular, the expression of iNOS has been associated with chondrocytes during the pathogenesis of OA (Grabowski *et al.,* 1997) and the overproduction of nitric oxide was detected in synovial tissue and articular cartilage (Melhiorri *et al.*, 1998). Mendes and collegaues., however demonstrated that H_2O_2 on its own cannot induce iNOS in human articular chondrocyte, but rather mediates IL-1β-induced iNOS expression and activation (Mendes *et al.*, 2003 a,b). However, in preliminary studies, H_2O_2 failed to cause significant induction in the release of IL-1β. Therefore the ability of C-20/A4 chondrocytes to release nitrate following H_2O_2 activation is not in agreement with previously published work by Mendes *et al* (Mendes *et al.*, 2003 a,b) and could thus be a characteristic of our cell-line.

Inflammation is a characteristic feature of osteoarthrtis (Hegemann *et al.*, 2005, Maccoux *et al.*, 2007) and the role of TNF-α, as inflammation-propagator is well documented (Oppenheim *et al.*, 1989; Pelletier *et al.*, 1991; Westacott *et al.*, 1996; Martel-Pelletier *et al.*, 1999). TNF-α receptors have been detected on the surface of human articular chondrocytes with expression levels significantly increased in osteoarthritic compared to healthy tissue (Westacott *et al.*, 1994). This thesis demonstrates that TNF-α can activate C-20/A4 chondrocytic iNOS to produce NO in amounts significantly higher than those detected in non-stimulated counterparts. The NO production was maximal following 80.0 pg/ml

but was not significantly different from those observed following stimulation with lower concentrations of the cytokine. This ability of TNF-α to induce nitrite release agrees with findings obtained from TN-α-stimulated canine chondrocytes, where human recombinant TNF-α up-regulates *iNOS* expression (Rai *et al.*, 2008). It was concluded that TNF-α was able to induce nitric oxide synthesis in chondrocytes, suggesting a possible role of this cytokine in chondrocyte-induced inflammation and possibly matrix metalloprotienase induction.

To further our understanding of the most appropriate stimulus for activation of C-20/A4 chondrocytes the effect of LPS (the principle component of the outer membrane of Gram-negative bacteria) was determined, as cells have been shown to generally respond to LPS by up-regulating expression of variety of inflammatory cytokines and cytotoxic mediators, such as NO, observed in arthritis (Haglund *et al.*, 2008, Campo *et al.*, 2010). C-20/A4 chondrocyte stimulation by various concentrations of TNF-α and LPS, led to significantly elevated NO production, an event accompanied by a concentration-dependent increase in chondrocyte death. The effect of TNF-α and LPS on this parameter was modest but sufficient for the purposes of this study, thus making them suitable for further evaluation in this study.

Given the role that MSU crystals play in gouty arthrirtis (McCarty *et al.*, 1966, McGill and Dieppe, 1991, Chilappa *et al.*, 2010) by depositing into the knee joint and driving leukocyte migration and the release of pro-inflammatory cytokines including IL-1β and KC in mouse (Getting *et al.*, 2006), their effect on chondrocytes was investigated. However, MSU crystals were unable to activate these cells at any of the concentrations tested, since a non-significant amount of nitrite release and pro-inflammatory cytokines and chemokines were detected To date no studies have shown that this cell line can be activated by MSU crystals, although a recent study has shown that MSU crystals can cause an upregulation in COX-2 in primary chondrocytes (Lee et al., 2009). The lack of effect could be due to differences often observed between primary and immortal cell-lines. However, although primarily thought to be the causative agent for gouty arthritis, there is conflicting evidence over the role they play in OA with a suggestion that urate crystals play an important role in contributing to the onset and/or acceleration of OA (for review see Nowatzky *et al.*, 2010).

Osteoarthritis is accompanied by extended apoptotic chondrocyte death, which has been thought to play a central role in the initiation and progression of the disorder (Blanco *et al.,* 1998; Kim *et al.,* 2000; Aigner *et al.,* 2001). A diverse set of stimuli can trigger the apoptotic process in virtually all eukaryotic cells (Steller, 1995, Thompson, 1995); therefore the cytotoxicity of various concentrations of H_2O_2, TNF-α, LPS and MSU on C-20/A4 chondrocytes via the mitochondrial functionality assay, MTT, which is a convenient assay used to monitor cell death quantitatively rather than qualitatively was investigated. H_2O_2, caused an 80 to 95 % reduction in cell-vialbility, even at the lowest concentration used. The reduction in chondrocyte cell-viability due to the endogenous production of excessive amounts of ROS has been previously described (Del Carlo and Loeser, 2002). In addition, H_2O_2 stimulation in C-20/A4 chondrocyte model provides data relevant to chondrocyte death initiated by endogenous ROS production, which has been reported to occur after mechanical trauma (Kurz *et al*., 2004). The use of this stimulus validated that C-20/A4 chondrocytes were able to respond to oxidative stress; however its use was discontinued for the present study due to the amount of cell death determined. Given the high-level of cell-death attributed to using H_2O_2, the effect of TNF-α on this parameter was determined. TNF-α led to a marked concentration-dependent cell death, causing chondrocytes to possibly undergo cell death through recruitment of the mitochondrial pathway of apoptosis thought blc2 family (Thomas *et al*., 2000). Its cytotoxicity was highest at 80.0 pg/ml, leading to a near 30 % reduction in cell viability, whilst lower concentrations, such as 60.0 pg/ml led to a more moderate 15 % decrease in cell viability and apoptosis. Moreover, the ability of TNF-α to trigger apoptosis in chondrocytes has been previously confirmed (Li *et al*., 2011). LPS stimulation caused ~20% decrease in chondrocyte viability, with lower concentrations (1.0 µg/ml) being more cytotoxic than higher concentrations (10.0 µg/ml). Taken together, these results demonstrate that both TNF-α and LPS impair mitochondrial function in C-20/A4 chondrocytes.

C-20/A4 chondrocytes respond to TNF-α and LPS by expressing and releasing pro-inflammatory cytokines.

Following the identification that C-20/A4 human chondrocytes respond to TNF-α and LPS, the effects of these inflammogens on the release of pro-inflammatory cytokines/chemokines, degradive enzymes and catabolites were evaluated since they are released from chondrocytes upon stimulation (Goldring *et al.*, 2011)

Initially, RT-PCR showed that following stimulation with TNF-α and LPS there was an upregulation in mRNA levels of *IL6* and *IL8* with TNF-α (60.0 pg/ml: a concentration chosen for sub-maximal release of nitrite and cell death) causing a marked increase in both *IL6* and *IL8* expression at all time points evaluated, with a peak at 2 h and decreasing thereafter. Upregulation of pro-inflammatory cytokines has been previously reported in this cell-line (Palmer *et al.*, 2002) and that this observation is translated to primary chondrocytes (Rai *et al.*, 2008). Given that these cells are in monolayers, the fast upregulation of these genes could be explained as they are exposed to the inflammogen in the cell culture media directly, so they can act immediately after stimulation (Rai *et al.,* 2008). After identifying the 6 h time-point as suitable for subsequent experiments, since it caused a sub-maximal up-regulation in IL-6 and IL-8 mRNA, different concentrations of TNF-α and LPS were evaluated. TNF-α caused a bell-shaped response with 60.0 pg/ml causing a sub-maximal upregulation in the cytokines genes. These results are in accordance with published data, where TNF-α has been shown to stimulate the production of variety of pro-inflammatory cytokines (Shinmei *et al.,* 1991; Shlopov *et al.,* 2000; Rai *et al.*, 2008)

Even though LPS has been used to induce cartilage degradation for the last 30 years (Jasin, 1983, Morales *et al.*, 1984, Tian *et al.*, 1989), the expression of its membrane receptor (TLR4) on chondrocytes and their response to LPS have only been recently investigated (Kim *et al.*, 2006; Haglund *et al.*, 2008). In order to gain a further insight into how chondrocytes respond to this pro-inflammatory stimulus, C-20/A4 chondrocytes were stimulated with increasing concentrations of LPS (0.1 – 1.0 µg/ml) and detected a concentration-dependent elevation in *IL-6* and *IL-8* expression, peaking at 1.0 µg/ml, which was in accordance with other studies (Bobacz *et al.*, 2007).

Following the identification of an upregulation in *IL-6* and *IL-8* mRNA, an ELISA assay was used to ascertain whether this message was genuinely translated into protein. IL-6 and IL-8 release was detected at 2, 6 and 24 h after stimulation with TNF-α and LPS and is in agreement with previous studies (Campo *et al.*, 2008, Henrotin *et al.*, 1996, Lotz *et al.*, 1992). For this part of the study, the concentration range of LPS was broadened by including 3.0 µg/ml and 10 µg/ml, in order to further investigate the effect of the concentration LPS on the production of these cytokines and to identify if a bell-shaped response could be observed. TNF-α and LPS caused a time and concentration-dependent effect with significant elevations in both IL-6 and IL-8 production. However, at 24 h post stimulation, all concentrations of LPS were equipotent, thus suggesting that a plateau might be reached at that time point. Previous studies have demonstrated that production of IL-1β, IL-6, IL-8, and TNF-α was responsive to a wide range of LPS concentrations (0.1 ng/ml – 10.0 µg/ml) and that these cytokines were first detected at 1 – 4 h and reached a plateau levels after 6 h (DeForge *et al.*, 1992).

The importance of these findings is related to the fact that the C-20/A4 cell-line responds to inflammatory stimuli in a similar fashion to primary cells/cartillage (Henrotin *et al.*, 1996; Campo *et al.*, 2008). Taken together, these findings demonstrated that the C-20/A4 chondrocytes are capable of expressing and producing IL-6, therefore suggesting that IL-6 may also play a physiological role in cartilage. An interesting finding of this study is the novel expression of *IL-8* by the human chondrocytic cell line, showing significant up-regulation following TNF-α-stimulation. Currently, the role of this chemokine has not been thoroughly investigated in human cartilage – a weak *IL-8* mRNA signal had been detected in normal untreated chondrocytes (Lotz, 1992; David, 2007) and freshly isolated chondrocytes have been shown to spontaneously release detectable amount of IL-8 (Fan *et al.*, 2005). Our data confirm these findings suggesting that IL-8 and other CXC chemokines, may trigger the release of matrix degradative enzymes and therefore the subsequent cartilage destruction (Lotz *et al.*, 1992).

In order to broaden our investigative spectrum of pro-inflammatory cytokines, we looked at IL-1β and MCP-1 mRNA and protein in response to TNF-α and LPS stimulation. These cytokines were chosen since IL-1β is active locally within cartilage and has been shown to play an important part in OA initiation and

progression (Fernandes, 1999 ; Kobayashi, 2005; Lopez-Armada, 2006; Kapoor, 2011). Moreover, IL-1β has been immunolocalized in chondrocytes taken from human OA cartilage with histologically confirmed degenerative changes (Dayer, 2002; Goldring *et al.,* 2011) with its role as propagator of inflammation leading to cartilage degradation being well documented (Westacott, 1996; Martel-Pelletier, 1999; Attur, 2000; Moldovan, 2000; Lopez-Armada, 2006; Kapoor, 2011).

MCP-1 evels were then determined following activation of C-20/A4 chondrocytes with varying concentrations of TNF-α and LPS. Although its main role is monocyte chemotaxis and activation (Zachariae *et al.*, 1990), it is involved in a vital pathologic relationship between chondrocytes and the synovium, where through the release of MCP-1, chondrocytes "invite" mononuclear phagocytes into the cartilage, resulting in rheumatoid pannus formation onto the articular surface (Villiger *et al.,* 1992). Here, TNF-α and LPS caused MCP-1 to be release with low levels of detection at early time-points and detectable amounts at 6 h. The time-frame for MCP-1 release observed in chondrocytes is supported by previous studies, which demonstrated that maximal production of MCP-1 was detected 4 h post induction of zymosan-peritonitis (Ajuebor *et al*., 1998). In addition, work by Lotz and colleagues, reveiled using *in situ* hybridization of cartilage organ cultures that chondrocytes in the superficial tangential zone responded within 2 h of stimulation with IL-1 by significantly increasing MCP-1 transcripts and chondrocytes in deeper layers responded by 4 h and reached maximum MCP-1 expression by 8 – 12 h (Villiger *et al.,*1992). This release of MCP-1 from chondrocytes highlights the fact that monocytes can migrate into the joint, therefore promoting cartilage degradation in the later stages of OA. Therfore, targetting these cytokines may play an important role in stopping the development of this pathology.

Following the identification of cytokine release from activated chondrocytes their effect on catilage degradation processes was determined (Brinckerhoff, 1992, Goldring *et al.*, 2008, 2011, Grassel *et al.*, 2009), consequential from the up-regulation and activation of MMPs (Shlopov, 1997, 1999, 2000). The effect of TNF-α and LPS on matrix degradative enzymes was investigated as these collagenases are unique enzymes capable of cleaving interstitial collagens

(Gadher *et al.*, 1990, Kevorkian *et al.*, 2004). Generally, collagenase expression is believed to be NF-κB dependent, a pathway that is also employed by the TLR receptors (Zhang *et al.*, 2008). TNF-α and LPS caused a concentration-dependent bell-shaped increase in *MMP1* and *MMP13* mRNA levels in C-20/A4 chondrocytes. These results are in agreement with a previous study, in which a four-fold increase in *MMP1* was was observed following stimulation with IL-1β (Grange *et al.*, 2006) although it is reported for the first time here that *MMP13* is also upregulated in these cells. This upregulation in *MMP1* and *MMP13* has previously been shown in primary chondrocytes (Shlopov *et al.*, 2000; Rai *et al.*, 2008). This was substantiated by the observation, that the levels of these collagenases increased with extended incubation times observed here and previously (Rai *et al.*, 2008). Interestingly, *MMP1* expression was significantly up-regulated in the stimulated samples, showing high similarity to the expression profile of *MMP1* in normal and OA human knee cartilage, and demonstrating the opposite of what takes place in the human hip during end stages of OA, where *MMP1* is down-regulated (Kevorkian *et al.*, 2004). However, the current increase in the expression could suggest a possible role of *MMP1* in this *in vitro* C-20/A4 chondrocyte model, and suggest that it resembles more the processes that occur in knee OA, rather than than that of the hip OA.

Statistically-significant changes were observed in the expression profile of *MMP3* with decreased expression, in a concentration-dependent manner, following a stimulation with both TNF-α and LPS for 6 h. This reduction in *MMP3* has not been previously reported in this cell-line and correlates with data in knee OA cartilage (Bau *et al.*, 2002). The down-regulation of *MMP3* in this *in vitro* model is interesting especially when compared to the reported increase in the *MMP3* levels in the synovial fluid of RA patients (Matsuno *et al.*, 2001). However, *MMP3* is reported to be the most highly expressed matrix metalloproteinase in normal knee cartilage, suggesting a maintenance function of this enzyme in normal cartilage metabolism, which was dysregulated in OA (Bau *et al.*, 2002; Kevorkian *et al.*, 2004).

Further examination of the collagenases showed that the main catabolic enzyme for type II collagen found in cartilage, *MMP13*, was increased in TNF-α-stimulated C-20/A4 chondrocytes, which is consistent with the current belief that

it is the principal collagenase in OA (Kevorkian *et al.*, 2004). Previous studies have demonstrated that IL-6 promotes cartilage degradation (Shlopov *et al.*, 2000) by directly inducing *MMP1* and *MMP13*. Here, its demonstrated that there is simultaneous up-regulation of *IL-6* and *MMP13*, therefore confirming the hypothesis that IL-6 and MMP13 are involved in the progression of OA. Overexpression of these genes in the cartilage may further induce an increase of hypertrophic chondrocytes (Goldring *et al.*, 2011, Tchetina *et al.*, 2005) resulting in the destruction of the upper-layer cartilage matrix and progression of cartilage degeneration.

In healthy articular cartilage, chondrocytes are actively maintaining the expression and ratio of collagens and proteoglycans (Hall, 1998). Chondrocytes are very sensitive to pro-inflammatory cytokines, an observation supported by this study. Studies have reported that pro-inflammatory cytokines either reduce or enhance the production of type II collagen, a marker of normal function of chodrocytes (Ho *et al.*, 2006). Therefore to determine if the cell-line responded in a similar fashion to primary cells, the direct effect of TNF-α on the expression of the cartilage specific collagens was determined by RT-PCR. TNF-α stimulation caused *COL1A1* and *COL2A1* levels to decline significantly, therefore inhibiting the chondrocyte compensatory synthesis pathways, required to restore integrity of the degraded matrix (Goldring and Goldring, 2004). It is important to point out, that TNF-α did not influence the differentiation indicator ratio of *COL2A1: COL1A1* detected in unstimulated C-20/A4 cells, which is an important observation as dedifferetiated cells would be undesirable for the purposes of this study.

To ensure that our *in vitro* cell-line system responded in a similar fashion to primary cells the effect of the glucocorticoid dexamethasone and the NSAID Indomethacin was evaluated given the role they play in the treatment of inflammatory pathologies. Glucocorticoids are powerful anti-inflammatory molecules shown to be able to repress transcriptional activation of genes including *IL1*, *IL-6, IL-8*, *TNF-α*, γ-interferon, colony stimulating factor (CSF)-1/macrophage, granulocyte macrophage (GM)-CSF (Taniguchi, 1988). Most of these genes are activated by the transcription factors NF-κB and AP-1, and their down-regulation confirms that glucocorticoids are interfering with these pathways

(Vayssiere *et al.,* 1997). NSAIDs exert their effects by inhibiting COX enzyme (part of the arachidonic acid cascade) and reducing prostaglandins leading to a diminished inflammation and pain (Vane, 1971, 1976). At present only COX-1 and COX-2 are clinically relevant, with COX-1 regarded as housekeeping enzyme responsible (via prostaglandins and thromboxane A$_2$) for physiological functions including protection of gut mucosal integrity and vascular homeostasis (Chen *et al*., 2008), whilst COX-2 appears to be a more important mediator of inflammation and thus a key factor in arthritic pain (Chen *et al*., 2008).

Dexamethasone and Indomethacin were administered 30 min prior to TNF-α stimulation of C-20/A4 chondrocytes. Dexamethasone and indomethacin have previously been shown to inhibit cytokine production in other cell systems (Mukaida *et al*., 1991). Glucocorticoids activate intracellular receptors that then bind to glucocorticoid-responsive elements in the promoters of various genes, or inhibit NF-κB transocation in the nucleus (Vayssiere *et al.,* 1997). Furthermore, they inhibit AP-1 DNA binding ability and therefore block the respective gene expression (Vayssiere *et al.,* 1997). In this study they were shown to inhibit TNF-α (60.0 pg/ml) induced expression of IL-6 and IL-8 mRNA and protein, an effect accompanied by a abrogation of the expression of *MMP1*, *MMP3* and *MMP13* over the time-course. This ability to modulate inflammatory pathways in this cell-type was in agreement with previous studies utilizing primary chondrocytes (Richardson and Dodge, 2003). Both the glucocorticoid dexamethasone and the NSAID indomethacin completely abrogated the production of IL-6 and IL-8 at all time points tested, and in doing so they negatively surpassed even the basal levels of production of these cytokines detected in unstimulated cells. However, although this effect may be desireable in management of acute inflammation, in chronic inflammatory diseases such as OA, this could lead to supression of the HPA axis and lead to impaired wound healing, Cushings syndrome and opportunistic infections (Gupta *et al.*, 2000; Alekseev *et al.*, 2001; Dorscheid *et al.,* 2006).

Data generated here looking at exempler cytokines, shows that TNF-α and LPS alone trigger a cascade of cytokines in this *in vitro* model. The results obtained from the ELISA and the PCR revealed a minimum 2-fold increase in inflammatory marker genes in these activated C-20/A4 chondrocytes. Of importance was the

observation that classical anti-inflammatory drugs (dexamethasone and indomethacin) were able to modulate these pathways. These data sugests that its possible to use this cell-line as a surrogate *in vitro* model for investigating inflammatory pathways within these cells and to evaluate the effects of the melanocortin peptides.

Melanocortin receptor expression in C-20/A4 chondrocyte and the role of melanocortins in modulation of pro-inflammatory cytokine production

Melanocortin peptides have potent antipyretic and anti-inflammatory effects (Grabbe, 1996; Getting *et al.,* 1999, 2001, 2008, 2009; Getting 2002, 2006, Luger, 2000), which they deliver via activation of a family of 7TM-GPCRs (Catania *et al.*, 2004). These are termed melanocortin receptors and to date five subtypes have been identified (Getting *et al.,* 2009). Previous research within the field of inflammation has highlighted that these peptides can modulate the effect of several pro-inflammatory cytokines and chemokines such as TNF-α, IL-1β, IL-6, and IL-8 (Catania *et al.*, 1999; Grassel *et al.*, 2009) and they are inducers of the anti-inflammatory cytokine IL-10 (Redondo *et al.,* 1998; Lam *et al.,* 2005) . This study tested the hypothesis that targeting melanocortin receptors may provide a novel therapeutic approach to treatment of chondrocyte inflammation, such as that observed in OA.

For this purpose, pharmacological and molecular techniques were used employing the melanocortin receptor pan-agonist α-MSH (Catania *et al.,* 2006; Getting, 2002, 2006; Getting *et al.,* 2009; Rajora *et al.,* 1996, 1997) and the selective MC_3 agonist D[TRP]^8-γ-MSH (Grieco, 2000; Getting, 2006; Getting *et al.*, 2008, 2009). To date, two receptors have been identified to mediate the anti-inflammatory effects of melanocortin peptides, namely the MC_1 and MC_3 (Getting, 2002, 2006, Getting *et al.,* 2009). However, some evidence points towards a role played by MC_5 in inflammation, since its expression has been detected on B-lymphocytes (Buggy, 1998) and T-lymphocytes (Taylor and Namba, 2001), suggesting a potential role in immuno-modulation.

Initially, expression of MC_1, MC_3 and MC_5 was determined in C-20/A4 chondrocytes, reported here for the first time with a strong signal MC_1 and MC_3, and a very faint signal corresponding to MC_5. Recently, gene expression of MC_1

was identified in the human chondrosarcoma cell line HTB-94 (Yoon *et al.,* 2008), and MC_1, MC_3 and MC_5 transcripts have been detected in primary articular chondrocytes (Grässel *et al.,* 2009). Whilst the detection of MC_1 is in agreement with previous studies, to our knowledge the detection of MC_3 expression in human chondrocytes is novel. Given the apparent disparity in the results observed and those generated previously in primary cells, western blotting was used to determine if mRNA for MC_1 and MC_3 was translated into protein.

Following the identification of mRNA and protein for $MC_{1,3}$ and mRNA for MC_5, the functionality of the receptors was determined by evaluating a panel of melanocortin peptides (displaying different receptor selectivity) on cAMP accumulation as detected by EIA. Melanocortin receptors are positively coupled to adenylate cyclase, which upon activation causes increase in intracellular cAMP formation (Catania *et al.,* 2006 ; Gantz *et al.*, 2003; Getting *et al.,* 2009). In order to determine the functionality of the melanocortin receptors, at first we tested whether the MC_1 receptor agonist α-MSH, the MC_3 selective agonist D[TRP]^8-γ-MSH, and the MC_5 selective agonists SHU9119, PG901 and PG911 could induce cAMP accumulation in the human C-20/A4 cells. Our functional studies showed that both α-MSH and D[TRP]^8-γ-MSH were able to ellicit a significant and concentration-dependent increase in cAMP formation, this effect being observed in C-20/A4 chondrocytes for the first time. Whilst α-MSH has previously been shown to induce cAMP accumulation in chondrocytes (Grassel *et al.,* 2009) the ability of the MC_3 agonist D[TRP]^8-γ-MSH to incude increases in cAMP has not been previously demostrated in chondrocytes. This increase in cAMP occurred in a bell-shaped manner and is in agreement with previous studies utilizing these peptides in other cell-types (Getting *et al.,* 2006).

Given that MC_5 mRNA was observed in C-20/A4 chondrocytes and has previously been shown to be epxressed on primary articular chondrocytes (Grassel *et al.,* 2009), the effect of selective MC_5 agonists PG901, PG911 (Grieco *et al.*, 2002) and the mixed agonist/antagonist SHU9119 (Hruby *et al.*, 1995) was evaluated. Treatment of cells with these peptides at all the concentrations evaluated did not cause an increase in cAMP accumulation. This lack of effect of the selective MC_5 agonists PG901 and PG911, was perhaps due

to the weak mRNA expression of this receptor, not translated to protein. Whilst we cannot completely rule out the expression of a functionally active MC_5 in the C-20/A4 cell model, given the fact that more selective compounds maybe developed in the future, which would allow further investigation of this receptor. At present, it is highly unlikely that this receptor plays a role in modulating the effects of the melanocortin peptides given the low expression level and lack of functionality displayed here in this model.

Given that α-MSH and D[TRP]^8-γ-MSH significantly elevated cAMP levels, the peptides were evaluated in the presence of the $MC_{3/4}$ anatagonist SHU9119 (Fan *et al.,* 1997) used at a concentration previously shown to inhibit the cAMP accumulation elicited by these peptides (Getting *et al.,* 2006), thus allowing identification of whether MC_1 or MC_3 was involved. Incubation of the C-20/A4 chondrocytes with SHU9119 was able to inhibit D[TRP]^8-γ-MSH confirming previous findings in other cell types (Getting *et al.,* 2006) and in models of inflammation (Getting *et al.*, 2008, Leoni *et al.*, 2008, Patel *et al.*, 2010), whereby D[TRP]^8-γ-MSH mediates its effects via MC_3. Not surprisingly, SHU9119 (10.0 µg/ml) failed to block α-MSH at all concentrations, except 3.0 µg/ml, which caused an extremely modest reduction in cAMP levels. At MC_3, the peptides ACTH$_{1-39}$, α-MSH, β-MSH and γ-MSH are equipotent (Getting, 2006) and even though α-MSH preferentially activates MC_1, it cannot be excluded that, at this concentration, some of the increase in cAMP observed with α-MSH may be inpart due to activation of the MC_3.

Numerous studies have highlighted the ability of melanocortin peptides to inhibit cytokine release *in vitro* (Lam *et al.*, 2005, 2006) and also *in vivo* models of inflammation (Ceriani *et al.*, 1994, Getting, 2002, Getting *et al.*, 2003, 2006, 2008). However, to date only one study has investigated the effect of melanocortin peptides on chondrocytes (Grässel *et al.*, 2009) and none using the selective MC_3 agonist D[TRP]^8-γ-MSH. C-20/A4 chondrocytes were treated with α-MSH (3.0 µg/ml), D[TRP]^8-γ-MSH (3.0 µg/ml), and SHU9119 (10.0 µg/ml) to ascertain their effect on basal release of cytokines from these cells. In order to determine the effect of the petides in the absence of detectible inflammation, IL-

1β, IL-6, IL-8 and MCP-1 levels were determind by ELISA and showed that none of the peptides caused an elevation in basal cytokine release from these cells.

The effect of melanocortin peptides on TNF-α induced inflammatory markers, was then evaluated since its has been shown to be involved in activation of chondrocytes leading to the degradation of cartilage within the knee joint. α-MSH has long been known to suppress inflammation by down-regulating the expression of pro-inflammatory cytokines, and to have anti-inflammatory and immuno-modulatory actions in rodent models of inflammation in a corticosterone-independent manner (Getting *et al.,* 1999). Here it inhibited TNF-α-induced release of IL-1β, IL-6, IL-8 and MCP-1 from C-20/A4 chondrocytes in a concentration-dependent manner. The peptide displayed potent anti-cytokine effects at both 2, and 6 h, a similar observation that was noted in primary murine peritoneal macrophages (Getting *et al.,* 1999) and macrophage cell-lines (Lam *et al.,* 2006). The peptide did not elicit any effect following 24 h incubation. The suppression of IL-1β, IL-6 and IL-8 by α-MSH in C-20/A4 chondrocytes is in accordance with the overall anti-inflammatory and protective capacity of the peptide (Catania *et al*., 2004).

Given the elevation in cAMP observed with the MC_3 agonist D[TRP^8]-γ-MSH, it was evaluated in this model. Here the effect of D[TRP]^8-γ-MSH (0.1 – 30.0 µg/ml) on modulation of pro-inflammatory cytokine release from human C-20/A4 chondrocytes activated by TNF-α was determined. C-20/A4 chondrocytes were treated with the selective melanocortin peptide for 30 min prior to stimulation with TNF-α, and subsequently incubated for 2-24 h, when supernatants were collected and analysed for IL-1β, IL-6 and IL-8 release. D[TRP]^8-γ-MSH showed a bell-shaped inhibition of IL-1β release with 3.0 µg/ml and 10.0 µg/ml being consistently the most potent concentrations, causing ~ 70-80 % inhibition. Similarly, a bell-shaped response in IL-6 was observed following 2 and 6 h of incubation, peaking at 3.0 µg/ml D[TRP]^8-γ-MSH, however, when the treatment was continued for 24 h, the peptide showed a concentration dependent inhibition of IL-6. IL-8 production was inhibited by D[TRP]^8-γ-MSH in a concentration-dependent manner at all time points, with 10.0 and 30.0 µg/ml being the most potent with a 70-80% reduction in this chemokine following TNF-α stimulation.

These data highlight for the first time the ability of this peptide to inhibit cytokine release from chondrocytes. The peptide displayed efficacy at all time-points evaluated and was still active at 24 h post treatment, correlating with the findings of this peptide in urate crystal-induced inflammatory cytokine release from macrophages (Lam *et al.,* 2005) The maximal anti-inflammatory effect at 2 and 6 h post stimulation was reached by 3 and 10.0 µg/ml of D[TRP]^8-γ-MSH concentrations previously shown to be effective (Getting *et al*., 2006, 2008) with a 70-80 % inhibition of IL-1β release.

This ability of the peptide to supress cytokine release was recently confirmed in a model of LPS-induced lung inflammation (Getting *et al.,* 2008). With respect to models of arthritis α-MSH has been shown to repress experimental adjuvant-induced arthritis in rats (Ceriani *et al.,* 1994), whilst a recent study by the Perretti group has highlighted the importance of the MC$_3$ agonist D[TRP]^8-γ-MSH in a model of serum transfer arthritis where the peptide was effective in wild type mice but not in MC3-/- null mice (Patel *et al.,* 2010). However, neither of the selective MC$_5$ agonists (MC$_{3/4}$ antagonists) PG901 and PG911 was able to cause significant decrease of TNF-α-induced pro-inflammatory cytokines, which together with the inability of the peptides to elicit cAMP increases, suggesting that this receptor is not functionally active in C-20/A4 chondrocytes.

The anti-inflammatory effects of α-MSH and D[TRP]^8-γ-MSH were evaluated on *IL-6* and *IL-8* transcription levels in the presence or absence of the MC$_{3/4}$ antagonist SHU9119, at a dose previously shown to abrogate the inhibitory effects of γ$_2$-MSH on chemokine release (10.0 µg/ml) (Getting and Perretti, 2000). α-MSH has been shown previously shown to suppress an array of inflammatory cytokines including TNF-α (Rajora, 1997 ; Delgado Hernandez, 1999) and IL-1β (Getting *et al*., 2003). α-MSH has tremendous effect on chemotaxis, further supported by the finding of Luger's group that this melanocortin peptide inhibits the production and release of IL-8 (Brzoska *et al*., 1999). Our study demonstrates and confirms the anti-inflammatory effect of not only α-MSH, but also D[TRP]^8-γ-MSH in human C-20/A4 chondrocytes. The pre-treatment of C-20/A4 chondrocytes with α-MSH (3.0 µg/ml) prior to TNF-α stimulation caused an ~30 % inhibition in the expression of *IL-6* (35 %) and *IL-8*

(25 %), therefore supporting the collected evidence that this peptide and its putative receptor have marked impact on IL-6 and IL-8 regulation. Additionally, α-MSH not only inhibited the transcription of these genes, but also the protein release of IL-6, IL-8 and MCP-1 from the cells. Cytokine ELISAs showed that there was a 70 %, 60 % and 21 % reduction in IL-6, IL-8 and MCP-1 protein levels released from C-20/A4 chondrocytes. The ability of α-MSH to exert anti-inflammatory actions has been well documented (Martin, 1991 ;Lipton, 1997, 1999; Catania, 1999), whereas the use of the mixed $MC_{3/4}$ antagonist SHU9119 (Hruby *et al.*, 1995; Fan *et al.*, 1997; Getting *et al.*, 2006) did not affect the observed anti-cytokine effects of α-MSH, suggesting that the latter must be preferentially activating MC_1 in order to exert its effect.

D[TRP]^8-γ-MSH was also able to diminish pro-inflammatory cytokines expression causing 27% and 36% inhibition of TNF-α-stimulated *IL-6* and *IL-8* transcription, respectively. Furthermore, similarly to the action of α-MSH, D[TRP]^8-γ-MSH led to 68 %, 45 % and 26 % reduction in IL-6, IL-8 and MCP-1 production, respectively, an observation in accordance with other studies exemplifying the anti-inflammatory and anti-migratory effects of D[TRP]^8-γ-MSH on cultured Mø both *in vitro* and *in vivo* (Getting *et al.*, 2006). An important finding was that SHU9119 completely obliterated the effect of D[TRP]^8-γ-MSH not only on cytokine transcription levels, but also on the synthesis and release of IL-6, IL-8 and MCP-1 from C-20/A4 cells compared to the effect of D[TRP]^8-γ-MSH alone. These data, together with the functional studies of MC_1 and MC_3 receptor activation, highlight not only the anti-inflammatory properties of α-MSH and D[TRP]^8-γ-MSH, but also confirms that both MC_1 and MC_3 might be the main targets for inflammation modulation in C-20/A4 chondrocytic system. Of interest is the fact that unlike dexamethasone and indomethacin, neither of the melanocortin peptides caused a complete abrogation of pro-inflammatory cytokine production. In contrast, they modulated the production of the tested cytokines, therefore allowing for some level of synthesis from the chondrocytes.

A novel finding of this study is the expression of functionally active MC_3 on C-20/A4 chondrocytes and that agonism of this receptor modulates the inflammatory response of TNF-α-activated human C-20/A4 chondrocytes.

However in this cell-line no one has ever investigated the effect of melanocortin peptides or their receptors on matrix metalloproteinases expression following TNF-α stimulation. We have shown that TNF-α potently up-regulates the expression of *MMP1* and *13* and that there is an upregulation in pro-inflammatory cytokines confirming previous findings (Fernandes *et al.*, 2002; Martel-Pelletier *et al.*, 1999). α-MSH pre-treatment of TNF-α-activated C-20/A4 chondrocytes led to a marked 48 % reduction in transcription of *MMP1*, one of the interstitial collagenases, significantly up-regulated in human osteoarthritic cartilage compared to healthy tissue (Reboul *et al.*, 1996; Kevorkian *et al.*, 2004).

Collagenase 3, or MMP13, is highly up-regulated in chondrocytes isolated from human osteoarthritic chondrocytes (Shlopov *et al.*, 1997) and following TNF-α stimulation (Rai *et al.*, 2008), and was significantly down regulated by α-MSH (3.0 µg/ml) in our model of TNF-α-activated C-20/A4 chondrocytes. The melanocortin peptide down-regulated the expression of *MMP13* by 67 %, compared to TNF-α-stimulated levels. These findings are in agreement with a recent study showing that α-MSH can inhibit TNFα-induced *MMP13* expression in the chondrosarcoma cell line HTB-94 (Yoon *et al.,* 2008).

In order to additionally confirm the involvement of MC_1 and MC_3 in the transmission of these effects, SHU9119 was added in conjunction with α-MSH, but no significant effect was observed on the expression of either *MMP1* or *MMP3*. However, an interesting observation was made when analysing the effect of this combination on *MMP13* expression. RT-PCR showed that SHU9119 and α-MSH synergistically inhibited the expression of this collagenase, leading to 87 % reduction compared to TNF-α-stimulated levels, thereby suggesting other mechanism by which this combination might affect the expression of this particular collagenase.

Similar results were obtained following pre-treatment of chondrocytes with the selective MC_3 agonist. D[TRP]^8-γ-MSH was more potent than α-MSH in reducing MMPs expression, with reductions of 89 %, 76 % and 92 % in *MMP1*, *MMP3* and *MMP13* expression, respectively, compared to levels detected following TNF-α treatment. However, when cells were treated with SHU9119 and D[TRP]^8-γ-MSH,

the effect was completely attenuated in all cases, confirming the involvement of MC_3 in the modulation of degradative matrix metalloproteinases. All these actions contribute to a local attenuation of the host's inflammatory response. Given the wealth of knowledge generated so far, few studies have looked at the potential of the melanocortins in inducing anti-inflammatory mediators in chondrocytes (Iannone *et al.*, 2001; Fernandes *et al.*, 2002).

In contrast to the suppressive effects of endogenous melanocortin peptides on pro-inflammatory cytokines production and release, they have been shown to elicit significant elevations in the production of the anti-inflammatory cytokine with potent immuno-suppressive properties, IL-10 (Bhardwaj *et al.*, 1996; Redondo *et al.*, 1998; Lam *et al.*, 2006). The importance of IL-10 in melanocortin receptor biology was first demonstrated in a model of contact hypersensitivity, where an antibody against this cytokine abrogated the protective action attained by α-MSH application (Grabbe *et al.*, 1996). The anti-inflammatory effect of α-MSH observed here and in other models, could be dependent on IL-10 induction, given that α-MSH was inactive in IL-10 knock out mice in a murine model of allergic airway inflammation (Raap *et al.*, 2003). This study sought to reveal, whether α-MSH and D[TRP]^8-γ-MSH could stimulate the production of IL-10 in C-20/A4 chondrocytes in the presence and absence of TNF-α. Additionally, the ability of SHU9119, PG901 and PG911 was tested in order to investigate whether these peptides could stimulate their cognate receptor (MC_5) to induce IL-10 synthesis. Human chondrocytes from healthy and osteoarthritic cartilage have been shown to express the anti-inflammatory cytokine IL-10 and its putative receptor IL10R (Iannone *et al.*, 2001), which upon interacting down-regulate TNF-α-induced *MMP1* and *MMP13* (Shlopov *et al.*, 2000). Here, we have demonstrated, that treatment with α-MSH and D[TRP]^8-γ-MSH (3.0 µg/ml), but not SHU9119 (10.0 µg/ml), PG901 and PG911 (3.0 pg/ml) leads to significant increase in basal IL-10 release from C-20/A4 chondrocytes. In addition, it was apparent that the response of the chondrocytes was time-dependent, with both α-MSH and D[TRP]^8-γ-MSH causing maximal induction of IL-10 at 6 h post-stimulation. α-MSH was significantly more potent than D[TRP]^8-γ-MSH at inducingIL-10 although both peptides elevated basal levels of IL-10 as early as 2 h post-administration, and maintained them for 24 h. These results demonstrate that in

part melanocortin peptides can exert a homeostatic control over chondrocyte physiology with the ability to induce anti-inflammatory cytokines. This therefore suggests a possible role in modulating basal levels of pro-inflammatory synthesis in this cell type even when no apparent inflammation is occurring.

Given the induction of basal IL-10 by the melanocortin peptides, their effect were investigated over a concentration range in the presence of TNF-α-induced chondrocyte inflammation over a time-course. α-MSH caused a concentration-dependent bell shaped response with maximal release of IL-10 caused by 1.0 µg/ml at early time-points and 10.0 µg/ml at later time points (24 h), whilst D[TRP]^8-γ-MSH (30.0 µg/ml) caused a maximal release of IL-10 at 2 h post-stimulation. The ability of α-MSH and D[TRP]^8-γ-MSH to trigger the production of IL-10 clearly suggests that activated melanocortin receptors may have crucial anti-inflammatory properties, conducted through activation of this cytokine.

The anti-inflammatory protein HO-1 has been implicated in the protection against tissue injury and is modulated by cytokines such as TNF-α (Fernandes *et al.*, 2003). It has been shown to be expressed and functionally active in human osteoarthritic chondrocytes from OA tissue with IL-10 shown to modulate its production (Lee and Chau, 2002; Fernandes *et al.*, 2003). A potential link between melanocortin receptor-dependent cAMP formation and HO-1 induction has previously been identified (Lam *et al.*, 2005). This idea stemmed from the fact that cAMP analogues have been shown to induce HO-1 in rat hepatocyte culture (Immenschuh *et al.*, 1998). To address this, the human C-20/A4 chondrocytes were employed to monitor alteration in HO-1 protein production, following incubation with α-MSH and D[TRP]^8-γ-MSH at 3.0 µg/ml in the presence and absence of TNF-α. C-20/A4 chondrocytes produced detectable basal levels of HO-1, and melanocortin peptides were able to provoke a marked up-regulation of HO-1 evident at 6 h post-incubation with the melanocortin receptor pan-agonist α-MSH and the synthetic MC$_{3/4}$ agonist D[TRP]^8-γ-MSH. Interestingly, when chondrocytes were pre-treated with the melanocortin peptides prior to stimulation with TNF-α, there was notable elevation in HO-1 production. Together with the fact that TNF-α moderately, but significantly induced the production of HO-1, these results are in accordance with published data

(Wagener *et al*., 2003). The downstream sequence of events currently remains unclear, and further investigation would be needed to elucidate the action of melanocortin receptor signalling in chondrocytes.

Chondrocyte apoptosis and the protective effect of melanocortin peptides

Chondrocytes are the only cell type present within articular cartilage and thus chondrocyte apoptosis plays an important part during the processes of cartilage development, aging and in cartilage pathologies (Blanco *et al*., 1998). Chondrocytes have been shown to be susceptible to endogenous degradative stimuli, such as TNF-α and IL-1β by up-regulating the synthesis of pro-inflammatory cytokines and matrix metalloproteinases, inhibiting collagen and proteoglycan synthesis, therefore causing loss of cartilage (Ismail *et al*., 1992; Martel-Pelletier *et al*., 1999; Fernandes *et al*., 2002; Kapoor *et al*., 2011). The importance of apoptosis has been identified with an increase in the number of apoptotic chondrocytes in osteoarthritic lesional than in non-lesional cartilage (Kim *et al*., 1999; Kim *et al*., 2000; Kouri *et al*., 2000; Hashimoto *et al*., 1998; Kirsch *et al.,* 2000). In addition, chondrocyte apoptosis and the reduction of tissue cellularity represent an important step in the pathogenesis of cartilage degradation (Blanco *et al*., 1998; Maneiro *et al*., 2003).

Currently, it remains unclear which pathways induce apoptosis and are responsible for the loss of chondrocytes and subsequent cartilage degradation. DeWolf and colleagues observed that TNF-α (30.0 ng/ml) stimulated caspase-3 driven apoptosis in human chondrocytic cell line (Nuttal *et al*., 2000) and that TNF-α induced apoptosis of bovine chondrocytes *in vitro* (Schuerwegh *et al*., 2003). In our *in vitro* study, we demonstrate that TNF-α potently induces chondrocyte apoptosis, suggesting that this is part of the mechanism of cartilage destruction, thereby substantiating those existing data. TNF-α concentrations (60.0 – 80.0 pg/ml) caused approximately 28% of the C-20/A4 chondrocytes to die, whereas lower concentrations caused around 10-15 % rate. Clinical studies have demonstrated that pathophysiological concentrations of TNF-α detected in OA synovium of patients with severe disease progression are in the range of 1.0 – 10.0 ng/ml (Westacott *et al*., 1990), much higher than the concentrations used

in this study. One reason for using lower concentrations is that chondrocytes *in vivo* die in the context of extracellular matrix, which may physically limit the levels of pro-inflammatory cytokines reaching the chondrocytes as opposed to *in vitro* experiments, where the chondrocytes are cultured in monolayer, allowing for fast and equal distribution of TNF-α to all chondrocytes, thereby increasing susceptibility of the chondrocyte to undergo apoptosis in response to TNF-α.

Moreover, the expression of p55 TNF-α receptor has also been localized in areas of osteoarthritic lesions of human cartilage (Webb *et al*., 1997), and given that the pro-inflammatory cytokine TNF-α is particularly important in the pathophysiology of cartilage disease, I aimed to further investigate the precise role it plays in chondrocyte apoptosis. It was confirmed that TNF-α modulates the activation of apoptotic pathways in human C-20/A4 chondrocytes and may be partially dependent on the activation of caspase-3 and -7. Western blot analysis showed that there was a significant 24 % increase in the protein levels of the activated executioner caspase-3 (Asp-175; 17, 19 kDa) following treatment of the C-20/A4 chondrocytes with TNF-α for 6 h. In addition, TNF-α treatment (60.0 pg/ml; 6 h) led to marked 5.7-fold increase in caspase-3/7 activities, which was confirmed by Caspase-Glo® 3/7 assay analysis. These results are an important finding and are not in accordance with the work by the Blanco group, who detected increased mRNA and protein levels of both caspase-3 and -7 in cultured human OA chondrocytes stimulated with TNF-α, but protein analysis detected only the intermediate, inactive forms of these enzymes (Lopez-Armada *et al*., 2006). Contrary to those reports, recent studies have demonstrated that TNF-α causes enhanced chondrocyte apoptosis by increasing capsase-3/7 activities (Nuttal *et al*., 2000; John *et al*., 2007; Kayal *et al*., 2010).

Role of melanocortins in prevention of pro-inflammatory cytokine-induced apoptosis.

Following the identification of α-MSH's and D[TRP]^8-γ-MSH's ability to markedly reduce the synthesis and production of pro-inflammatory cytokines, as well as down-regulating degradative matrix metalloproteinases expression, their effect on TNF-α induced cellular toxicity and cell-death inducing signals was evaluated. Both peptides inhibited TNF-α-induced cell death and significantly down-

regulated the production and activity of caspase-3 and -7. Both peptides at all concentrations tested failed to cause any damage to the treated C-20/A4 chondrocytes and additionally caused 40 - 50 % reduction in cleaved caspase-3 protein expression as determined by western blot. These results were confirmed by testing the activity of the executioner caspases 3 and 7 and measuring mitochondrial functionality following pre-treatment with α-MSH and D[TRP]^8-γ-MSH on TNF-α-activated C-20/A4 chondrocytes. The peptides exhibited strong concentration-dependent protective effect against TNF-α-induced cell death, whereby 49 % and 55 % reduction in chondrocyte apoptosis was observed following treatment with α-MSH (3.0 μg/ml) and D[TRP]^8-γ-MSH respectively.

Other studies support the molecular mechanism by which melanocortin peptides prevent apoptosis in chondrocytes, with one particular study on neuronal cell-line GT1-I demonstrating the inhibitory effect of the melanocortin peptide NDP-MSH on caspase-3 activation as readout of apoptosis (Windebank *et al*., 1994) whilst α-MSH prevents LPS/INF-γ-induced astrocyte apoptosis via activation of MC$_4$ (Caruso *et al*., 2007).

To our knowledge, this study is the first to show the inhibitory effect of α-MSH and D[TRP]^8-γ-MSH in a model of TNF-α-induced chondrocyte apoptosis *in vitro*. Through antagonism of MC$_{3/4}$, demonstrates that MC$_1$ and MC$_3$ are involved in the transmission of the anti-apoptotic effects of α-MSH and D[TRP]^8-γ-MSH respectively in the human C-20/A4 chondrocytic cell line. SHU9119 (10.0 μg/ml) antagonized the effects of D[TRP]^8-γ-MSH, but not α-MSH on down-regulating the production of cleaved caspase-3 as a marker of apoptosis and consistently reversed the protective effect of the selective MC$_3$ agonist on mitochondrial function in the model of TNF-α-induced chondrocyte death. The combination led to 27 % up-regulation of cleaved caspase-3 production and cell death was observed at TNF-α-produced levels regardless of the concentration used.

Combined, these results demonstrate, that MC$_3$ is particularly important in transmitting the anti-inflammatory, cyto-protective, anti-apoptotic and immuno-modulatory effects of melanocortin peptides. In addition, the role of MC$_1$ has been also confirmed, given the fact that MC$_5$ is non-functional in this C-20/A4 chondrocyte model and MC$_2$ is solely activated by ACTH$_{1-39}$ (Getting, 2006).

Effect of hypotonic solution on chondrocyte function.

To further understand the potential role that melanocortin peptides play in modulating chondrocyte activity, their effect was investigated on the function of osmotically challenged chondrocytes. Articular cartilage is highly hydrated tissue whereby approximately 30 % of the water in the cartilage is found within the collagen intrafibrillar space (Hall, 1998). The amount of water in the cartilage depends on the fixed charge density of the proteoglycans, which bear strong negative electrical charges (Maroudas *et al*., 1979), neutralised by positive ions in the surrounding fluid. The high concentration of ions in the extracellular matrix, compared to the outside the tissue, has been shown to increase osmotic pressure (Maroudas, 1979; Maroudas and Evans, 1972). In OA and upon matrix degradation, the water content of cartilage increases and leads to over-hydration of the negatively charged proteoglycans, which alters the chondrocyte extracellular physio-chemical environment by reducing the osmolality causing an increase in cell volume; an early event during osteoarthritis (Gardner, 1992; Bush & Hall, 2004).

C-20/A4 chondrocytes were subjected to hypotonic conditions (280 to 140 mOsm), and a significant time-dependent up-regulation of pro-inflammatory cytokines *IL-6* and *IL-8* from C-20/A4 chondrocytes was observed, with a 37-fold increase in *IL-6* and 8-fold up-regulation of *IL-8* expression at 24 h and 42-fold and 10 fold, respectively at 72 h. These results were further substantiated by ELISA detection of these cytokines, which confirmed that the intensification on cytokine transcription, in response to chronic hypo-osmotic challenge, was translated into protein released from the C-20/A4 chondrocytes.

This study, to our knowledge, is the first to investigate the effect of lowered media osmolarity on C-20/A4 chondrocyte inflammatory profile. A novel finding, demonstrated by this work, is that upon reduction of extracellular osmolarity, C-20/A4 chondrocytes respond by increasing not only pro-inflammatory cytokines, but also the expression of *MMP1* and *MMP13*, which increased in a time-dependent manner. *MMP1* mRNA was up-regulated 2.3-fold following 24 h incubation in the 140 mOsm DMEM and this increased to 4-fold, compared to cells incubated in normal 280 mOsm culture media. Interestingly, *MMP13* mRNA

(which is not present in non-treated C-20/A4 or healthy human cartilage) was elevated following stimulation with 140 mOsm media, and the detected amounts were significantly higher compared to TNF-α-stimulated levels. Hypo-tonicity did not seem to affect *MMP3* expression in the first 24 h of incubation, but caused increased transcription when the chondrocytes were incubated for 72 h. This finding is supported by work on other cell type showing that cellular stresses such as pro-inflammatory cytokines and osmotic stress activate MAPK pathways (JNK, ERK1/2 and p38) (Lewis, 1998; Garrington, 1999). It has been shown that JNK and ERKs phosphorylate AP-1 family member c-Jun (Karin, 1995, Leppa *et al.,* 1998), which then dimerizes with c-Fos and initiates the transcription of various MMP genes. Other groups have shown that the ERK1/2 pathway mediates the activation of the *MMP1* promoter via an AP-1 element (Frost, 1994; Rutter, 1995; Korzus, 1997).

However, whether the altered osmolality of the C-20/A4 chondrocytes media is directly triggering the expression of these MMPs cannot be concluded by this work, as 140 mOsm DMEM also affects the synthesis of various cytokines, such as IL-6, which in turn can directly alter both *MMP1* and *MMP13* gene expression in a concentration-dependent manner. This is an important finding, since osteoarthritic cartilage is also defined by increased expression and synthesis of pro-inflammatory cytokines, matrix metalloproteinases and increased tissue hydration.

Since the events of OA, which we attempted to mimic here in the C-20/A4 chondrocyte model (increased production and release of pro-inflammatory cytokines and catabolic matrix metalloproteinases, increased degradation and reduced collagen type II production, chondrocyte apoptosis and cartilage hydration), it seems likely that a perpetuation of self-inducible and pathological events could lead to the chronic profile of this joint disorder, thus novel avenues for pharmacological intervention could look at targeting these processes.

The effect of α-MSH and D[TRP]^8-γ-MSH on hypo-tonicity induced over-expression of matrix metalloproteinases was determined. D[TRP]^8-γ-MSH down-regulated both *MMP1* and *MMP13* expression even at 24 h post stimulation with 140 mOsm DMEM. However, α-MSH only caused a modest non-significant

decrease in the transcription of *MMP1* and *MMP13*, its effect was not statistically different from the levels caused by the hypotonic media alone. None of the peptides altered the expression of *MMP3* by C-20/A4 chondrocytes.

These results suggest, that α-MSH probably due to its short half-life (6 h) is unable to down-regulate MMPs expression at 24 h, especially, since the hypo-osmotic medium, surrounding the chondrocytes seems to be exerting strong pro-inflammatory effects, which is unlike the effect of the pro-inflammatory stimuli used for various cell stimulation. The fact that IL-6 and IL-8 levels are increasing even at 72 h post incubation of C-20/A4 chondrocytes with 140 mOsm DMEM, suggested a possible role for these cytokines in the synergistic induction of MMPs expression at time-points as late as 24 and 72 h.

Primary bovine chondrocyte (P_0) activation by various pro-inflammatory stimuli.

Following the identification of the protective role that melanocortin peptides could play on pro-inflammatory cytokines and MMP expression following TNF$-\alpha$ stimulation in C-20/A4 chondrocytes, it was decided to determine whether these effects translated to primary cells and cartillage. C-20/A4 chondrocytes have been shown throughout this thesis to respond to various stimuli by secreting significant amounts of pro-inflammatory cytokines, chemokines and other non-cytokine pro-inflammatory mediators, such as NO and matrix metalloproteinases. Even though immortalized cell lines are a suitable method for studying the function and the response of chondrocytes to various stimuli, primary cultures of articular chondrocytes may respond differently to these stimuli. In order to elucidate whether the findings confirmed in the C-20/A4 cell-line chondrocytes are consistent with the responses detected within primary chondrocytes, we employed primary articular chondrocytes extracted from load bearing regions of bovine knee joint cartilage.

High-density primary articular chondrocytes cultures (passage 0, P_0) were established to study the effect of TNF-α and LPS on the release of pro-inflammatory cytokines IL-1β, IL-6, IL-8 and MCP-1. Primary chondrocytes reacted to both TNF-α and LPS via a concentration-dependent up-regulation of

the production and release of all cytokines measured. TNF-α at all concentrations induced significant IL-6 release, which was highly comparable to the response observed in C-20/A4 cells, with the exception of the highest concentration of TNF-α (which was less effective in C-20/A4 compared to P_0 primary chondrocytes). These results are in accordance with previous observations that IL-6 mRNA is up-regulated following TNF-α-stimulation of human articular chondrocytes (Shlopov *et al*., 2000), or in OA tissue (Hrycaj *et al.,* 1995, Shinmei *et al.,* 1991). In addition, LPS was similarly potent in the induction of IL-6 secretion from both C-20/A4 cells and primary articular chondrocytes, despite somewhat higher levels in C-20/A4 cells.

In addition, bovine articular chondrocytes, much like the C-20/A4 chondrocytes were readily inducible to release IL-8, which can promote neutrophil-mediated inflammation and cartilage degeneration. TNFα and LPS promoted abundant IL-8 secretion from the primary bovine chondrocytes, a finding that is in agreement with the ability of these inflammogens to exert the same effect in human articular cartilage (Terkeltaub *et al*., 1991). The maximal IL-8 release from bovine chondrocytes was comparatively low, reaching just 50.0 pg/ml (TNF-α 40.0 – 60.0 pg/ml), as opposed to 205 pg/ml of IL-8 detected in C-20/A4 chondrocytes following stimulation with the same concentrations of the cytokine. Regardless of these slight discrepancies, this study accentuates the fact that articular chondrocytes are readily inducible to express the *IL-8* gene and secrete biologically active IL-8, which can promote neutrophil-mediated inflammation and cartilage destruction.

TNF-α and LPS stimulated articular chondrocytes released significant amounts MCP-1, in concentrations concordant with the detected levels in C-20/A4 chondrocytes (maximum 120 pg/ml) following 6 h stimulation, thus confirming our initial findings. In agreement with the results of this study is the work by Lotz and colleagues, who have demonstrated the ability of human articular cartilage to respond to IL-1β stimulation by significantly up-regulating the transcription of *MCP1* within 2 – 4 h of stimulation (Villiger *et al*., 1992). The fact that chondrocytes release biologically active MCP-1 in response to factors that are present in cartilage or synovium of osteoarthritic patients provides a possible

mechanism by which chondrocytes implicate in the initiation and progression of cartilage degradation observed in osteoarthritis.

Il-1β has a fundamental function in osteoarthritis pathophysiology as it controls the degeneration of articular cartilage matrix and severely affects chondrocyte apoptosis. Therefore targeting the activation mechanism of this catabolic cytokine seems to be essential as a therapeutic approach (Moldovan *et al*., 2000; Lopez-Armada *et al*., 2006; Kapoor *et al*., 2011). In this study, we have already demonstrated that both TNF-α and LPS are able to induce moderate secretion of this cytokine from human C-20/A4 chondrocytes. These findings were further substantiated by the detection of IL-1β secretion in response to TNF-α and LPS from primary articular chondrocytes as well. In both cell types, TNF-α generally initiated low (up to 40.0 pg/ml) levels of IL-1β secretion.

As previously discussed, the expression and secretion of all these pro-inflammatory cytokines has been previously detected in chondrocytes extracted from osteoarthritic cartilage (Martel-Pelletier *et al*., 1999; Maccoux *et al*., 2007; Villiger *et al*., 1992), which shows the relevance of the results acquired by the present study.

Effect of melanocortin peptides on TNF-α-activated primary bovine articular chondrocytes.

During this study, we have shown that human C-20/A4 chondrocyte express functionally active melanocortin receptors MC$_1$ and MC$_3$, and show very slight expression of MC$_5$, which was found to be functionally inactive. Here we demonstrate the expression of MC$_1$ in bovine primary chondrocytes and yet again we detected MC$_5$ expression. The expression of MC$_3$ unfortunately, was not investigated, because at the time the experiments were conducted the full sequence of the bovine *MC$_3$* gene was not yet discovered, preventing the construction of primers.

Here, bovine articular chondrocytes consistently express *MC$_1$*, which is in agreement with other studies reporting the expression of this receptor in chondrocyte cell lines as well as primary cells (Yoon *et al.,* 2008; Grässel *et al.,* 2009). MC$_5$, which has also been detected, showed great variation between the

tested animals, with some samples showing slight expression, and others none at all. Additional work is required to fully unravel the expression of this melanocortin receptor in bovine primary chondrocytes.

To support our RT-PCR detection of the melanocortin receptors, we evaluated the pan agonist α-MSH, to test MC_1 functional activity, whilst the synthetic selective MC_3 agonist D[TRP]^8-γ-MSH was used to evaluate whether bovine chondrocytes possess a functionally active MC_3. The $MC_{3/4}$ antagonist and potent MC_1 agonist SHU9119 was also evaluated to determine 1) to determine whether D[TRP]^8-γ-MSH specifically activates its putative receptor (MC_3) by blocking its binding sites; and 2) to conclude the role of α-MSH and MC_1 in modulation pro-inflammatory cytokines levels in the primary articular chondrocytes (P_0).

Pre-treatment of primary chondrocytes with α-MSH and D[TRP]^8-γ-MSH prior to stimulation with TNF-α caused a concentration-dependent decrease in IL-1β a similar observation to that noted in the cell-line. However, a note should be taken that the inhibitory effect of α-MSH (1.0 and 3.0 µg/ml) on IL-1β concentrations (~ 40 % reduction) was lower in bovine chondrocytes compared to the cell-line, where similar concentrations caused > 80 % reduction.

Interestingly, lower concentrations of SHU9119 (1.0 and 3.0 µg/ml) led to a low 20 % inhibition in IL-1β release from bovine chondrocytes, suggesting a potential role for MC_1. However, SHU9119 was not capable of reducing either IL-6 or IL-8 production at any of the concentrations tested, which is in agreement with our findings from the human C-20/A4 chondrocytic cell-line. Conversely, both α-MSH and D[TRP]^8-γ-MSH led to marked concentration-dependent bell shaped decreases in both IL-6 and IL-8 production. Unlike the effect of α-MSH on IL-1β, 1.0 and 3.0 µg/ml of α-MSH abrogated IL-6 production from the cultured primary bovine chondrocytes, by 93 % and 87 % reduction respectively. Similarly, D[TRP]^8-γ-MSH (3.0 µg/ml) caused a similar degree of inhibition of IL-6, whilst both peptides inhibited IL-8 release, a similar observation as noted in the cell-line. This observation of bell-shaped inhibition of cytokine production by melanocortin peptides from primary cells was initially observed in macrophages following MSU crystal stimulation (Getting *et al.,* 1999)

The MC$_{3/4}$ antagonist SHU9119, did not significantly alter the anti-inflammatory effect elicited by α-MSH on TNF-α-induced IL-1β, IL-6 and IL-8 production. Interestingly, SHU9119 and α-MSH (30.0 µg/ml) appeared to be synergistically acting to reduce the production of IL-6. From the results, it seemed that, with increasing concentrations of α-MSH, it started to slowly lose its effectiveness, and thus a combination with SHU9119 allowed it to "help" the α-MSH (30.0 µg/ml) to maintain the same level of cytokine inhibition. These findings are controversial, and may suggest that: 1) α-MSH does not act through activation of MC$_3$ in chondrocytes; 2) SHU9119, being relatively selective MC$_5$ agonist might be causing very mild reduction in IL-6 levels, only if high concentrations of α-MSH are used. This might be a resulting from desensitization of the receptors on chondrocytes and possible internalisation of MC$_1$, allowing for or inducing a switch in the mechanisms by which the peptides works. It has been long known that GPCRs, such as melanocortin receptors, are regulated via multiple mechanisms(Clark, 1986, Perkins, 1991). This has been well illustrated by studies of β2-adrenergic receptor, reviewed in detail (Carman and Benovic, 1998, Ferguson *et al.*, 1998, Lefkowitz *et al.*, 1998), where agonist-induced activation of the receptor activates adenylate cyclase within seconds of binding (von Zastrow and Kobilka, 1992). However, following prolonged activation of the receptors, their ability to induce cAMP formation declined significantly. In addition, many GPCRs can be regulated by ligand-induced endocytosis or internalization (von Zastrow and Kobilka, 1992). SHU9119 (10.0 µg/ml) potently blocked and reversed the anti-inflammatory effect of D[TRP]^8-γ-MSH at all concentrations tested, thereby suggesting that MC$_3$ receptor is expressed and functionally active in bovine primary chondrocytes.

Given the ability of these peptides to modulate pro-inflammatory cytokines, the peptides were evaluated on production of pro-resolving anti-inflammatory cytokines. IL-10 production was observed from stimulated bovine primary chondrocytes and is in agreement with that observed in human chondrocytes from healthy and osteoarthritic cartilage, which express both IL-10 and its putative receptor IL10R (Iannone *et al.*, 2001). Consistent with previous findings α-MSH caused the release of IL-10 (Bhardwaj et al., 1996), the melanocortin peptides substantially elevated the production of the anti-inflammatory cytokine.

Primary bovine chondrocytes did not produce detectable basal IL-10 release; however, following 30 min pre-treatment of TNF-α-activated primary chondrocytes, there was a bell-shaped elevation of this cytokine following stimulation. Once again, SHU9119 failed to block the effect of α-MSH but completely abolished the anti-inflammatory effect of D[TRP]^8-γ-MSH, therefore indicating a dual role for both MC$_1$ and MC$_3$ in modulating the inflammatory response in these cells.

Cartilage impact and the anti-inflammatory and protective effect of melanocortins in models of mechanical trauma on cartilage tissue.

Cartilage metabolism is contingent in part with mechanical forces, including shear stress and hydrostatic pressure that occur during normal joint loading. Previous *in vitro* work confirms that chondrocytes in culture continue to respond to a variety of loading conditions (Mankin and Lippiello, 1970). The distinctive properties of cartilage are believed to affect the chondrocyte reaction to pressure. It is largely documented that within physiological limits mechanical loading of healthy joints contributes significantly to the maintenance of the articular cartilage ECM by chondrocytes, but the precise relationship between mechanical loading and chondrocyte metabolism is still vague (Saamanen *et al*., 1987). Mechanical loading above physiological ranges and/or frequency gives rise to substantial cartilage injury (Burton-Wurster *et al.,* 1993; Guilak *et al.,* 1994) including increase in synthesis and release of pro-inflammatory mediators and degradative enzymes (Pickvance *et al*., 1993, Guilak *et al*., 1994, Wang *et al*., 2010b). Alteration in joint loading is additionally considered an important factor in the initiation of osteoarthritis (Anderson and Felson, 1988).

The effect of mechanical trauma on the functionality and metabolism of chondrocytes is receiving increasing attention (D'Lima, 2001a,b; Borrelli, 2004; Kurz, 2004), particularly because within mature articular cartilage, chondrocytes do not undergo cell division (Sailor, 1996; Buckwalter, 1998). Additionally, osteoarthritis is featured by reduced cellularity (Stockwell, 1991), a fact that is thought to contribute to the inability of the remaining chondrocytes to maintain normal matrix synthesis, thereby contributing to cartilage degradation (Bush *et al*., 2005).

Given the importance of this in the development of OA, the effect of a single impact blunt mechanical impact, delivered via drop tower (Bush *et al*., 2005) on chondrocyte viability and rates of pro-inflammatory mediator synthesis were determined. Following impact *in situ* chondrocytes within the superficial zone were shown to be particularly sensitive to mechanical injury confirming previous observations using bovine articular cartilage explants (Bush *et al*., 2005). Chondrocyte cell death was apparent 6 h post impact and was visibly localized to the distinct areas of impact. Confocal laser scanning microscopy and imaging allowed us to study the cell viability of individual chondrocytes within bovine articular cartilage explants subjected to a single blunt impact.

The data showed that ~3 % of the chondrocytes in non-impacted cartilage were non-viable following excision of the explant from the joint and increased significantly to 13 % following application of mechanical injury to the cartilage explant. The experiments conducted in this study were performed on cartilage explants excised from the underlying bone. However, judging from the results and the confinement of chondrocyte damage to the superficial zone, it is unlikely that the subchondral bone is a major factor of cartilage surface properties within these experiments (Bush *et al*., 2005).

It is important to note that this study has not been designed to clarify *in vivo* response to single blunt impact. The drop tower is a useful technique for simulating true impact (Bush *et al.,* 2005), but it is not possible to mimic the impact waveform or displacement applied to the cartilage explant during compression. Nevertheless, the observed changes in matrix structure, surface damage to cartilage explant and the loss of chondrocytes in the superficial zone (Quinn *et al.,* 1998, Quinn *et al.,* 1999) are similar to the changes observed in osteoarthritic cartilage (Thompson *et al*., 1991; Wilder *et al*., 2002). Within this study, we have demonstrated the protective properties of melanocortin peptides in a model of TNF-α-induced chondrocyte apoptosis/cell-death. Here, we have shown that α-MSH (3.0 µg/ml), but not D[TRP]^8-γ-MSH or dexamethasone (1.0 µM) are able to inhibit basal chondrocyte apoptosis in non-impacted cartilage. The melanocortin receptor pan-agonist decreased chondrocyte cell-death from 3 % to <1 %.

In order to expand our study on the effect melanocortins on primary chondrocytes *in situ*, we detected basal levels of pro-inflammatory mediator release of IL-1β, IL-6 and IL-8 in non-impacted cartilage and compared them to the effect of melanocortin peptides and dexamethasone. Our data demonstrates that chondrocytes from non-impacted cartilage produce significant basal levels of IL-1β, IL-6 and IL-8 cytokines, consistent with the effect of TNF-α on the chondrocytic cell-line. Previously, freshly isolated chondrocytes have been shown to spontaneously release detectable amounts of IL-8, but these are rapidly increased following stimulation, suggesting that functionally expressed chemokines by chondrocytes may trigger the release of matrix degradative enzymes and subsequent cartilage destruction (Lotz *et al.*, 1992). IL-1β was also detected in cartilage explants prior to impact, similarly to that observed in normal human articular cartilage (Middleton *et al.*, 1996). Bovine articular chondrocytes *in situ* also produced IL-6, a potent pleuotropic cytokine and important mediator of the cell interactions in osteoarthritis. This was consistent with the finding of Shinmei and collegues, who detected the expression of this cytokine in human articular chondrocytes (Shinmei *et al.*, 1989). These basal release of cytokines were not modified by α-MSH, D[TRP]^8-γ-MSH or dexamethasone.

However, the peptides led to significant reduction of cell death when administered 30 min prior to single blunt impact inflicted by the drop tower. At 6 h post-impact, α-MSH and D[TRP]^8-γ-MSH caused significant protection of the chondrocytes from cell death, compared to levels detected following mechanical trauma in the absence of the peptide. Dexamethasone also caused a protective effect following impact although with a lower level of protection being observed compared to the peptides. Consistent with our previous observations that melanocortins can protect against TNF-α-induced chondrocyte apoptosis, these data confirms that melanocortins exert cyto-protective properties not only in cell-line chondrocytes *in vitro*, but also in primary articular chondrocytes *in situ*.

Mechanical injury was associated with a significant upregulation of IL-1β, IL-6 and IL-8 release compared to levels produced by chondrocytes in non-impacted cartilage, and the melanocortin peptides α-MSH, D[TRP]^8-γ-MSH and dexamethasone significantly down-regulated the release of these cytokines. α-

MSH was the least effective of the three treatments on cytokine release, whilst D[TRP]^8-γ-MSH was more effective than α-MSH in reducing these cytokines levels. Dexamethasone showed the greatest effect in reducing pro-inflammatory synthesis caused by mechanical trauma to the cartilage explants. Here, in accordance with our previous results, melanocortins exhibited modulatory effects on the production of pro-inflammatory cytokines from impacted cartilage, rather than complete abrogation of the cytokine response as previously seen in models of acute inflammation (Getting *et al.,* 1999, 2002, 2006, 2008). However, the data for dexamethasone was contradictory to the previous findings, showing that dexamethasone abrogated completely the release of these cytokines from cell-line chondrocytes. One explanation for these findings might be the relatively low permeability of the cartilage matrix, compared to culture media, which poses no physical barrier for the drugs to reach and affect chondrocytes and their function. Furthermore, pro-inflammatory mediator release from C-20/A4 cell line was caused by exogenous TNF-α-stimulation, whereas *in situ* articular chondrocytes were activated by mecanical trauma. Nevertheless, it is important to note that joint trauma leads to excessive synthesis of pro-inflammatory cytokines, one of the major ones being TNF-α (Ertel *et al.,* 1995), which could potentiate the cartilage destruction shown by our work on C-20/A4 chondrocytes.

Chapter 5
Conclusion and Future work

5.1 Conclusion.

The results generated in this thesis indicate that C-20/A4 chondrocytes and primary articular chondrocytes *in vitro* and *in situ* respond to exogenous stress (pro-inflammatory mediators, changes in extracellular osmolarity, mechanical trauma) by producing significant amounts of pro-inflammatory cytokines (such as IL-1β, IL-6, IL-8 and MCP-1), and collagen derisive matrix metalloproteinases (*MMP1* and *MMP13)*, therefore corroborating the possible role of the chondrocyte in initiation and progression of cartilage degradation, as observed in OA. In addition, the work presented here indicates that there is significant down regulation of cartilage ECM components such as collagen type I and type II upon TNF-α-activation of C-20/A4 chondrocytes, which if translated to *in vivo* work could additionally lead to a reduction in the quality of the cartilage matrix, thereby leaving the cartilage prone to injury.

Melanocortins have been previously shown to possess a vast range of physiological and pharmacological actions. However, little research has occurred with respect to their role in chondrocyte inflammation. This thesis highlights the anti-inflammatory and anti-apoptotic effects of melanocortin peptides α-MSH and D[TRP]^{8}-γ-MSH in an *in vitro* chondrocyte-based model of TNF-α-induced stimulation. The melanocortin peptides were able significantly down regulate pro-inflammatory cytokines and cartilage degradative MMPs, but also to lower cytokine-induced apoptosis and to induce the anti-inflammatory proteins (IL-10 and HO-1) in this cell-line. These effects were transmitted through the activation of MC$_1$ and MC$_3$, both of which were found to be expressed and functionally active in C-20/A4 chondrocytes. The influence of the peptides was not limited to the human cell-line chondrocytes; instead, the results highlight the ability of the peptides to reduce TNF-α-induced pro-inflammatory cytokine and MMP expression in primary bovine articular chondrocytes.

Moreover, the data presented here confirm that mechanical impact is an effective modulator of chondrocyte metabolism *in vitro*. This information adds to our basic understanding of how mechanical loading influences articular cartilage metabolism. Understanding the precise mechanisms by which impact trauma alters cartilage metabolism will provide vital insights for development of

approaches for the treatment of arthritic pathologies. The melanocortin peptides not only showed chondroprotective effect in C-20/A4 chondrocytes, but also in articular chondrocytes *in situ*. This is a novel finding, suggesting potential clinical implications whereby people with joint injury/trauma or people suffering from OA could benefit from potential melanocortin peptide treatment.
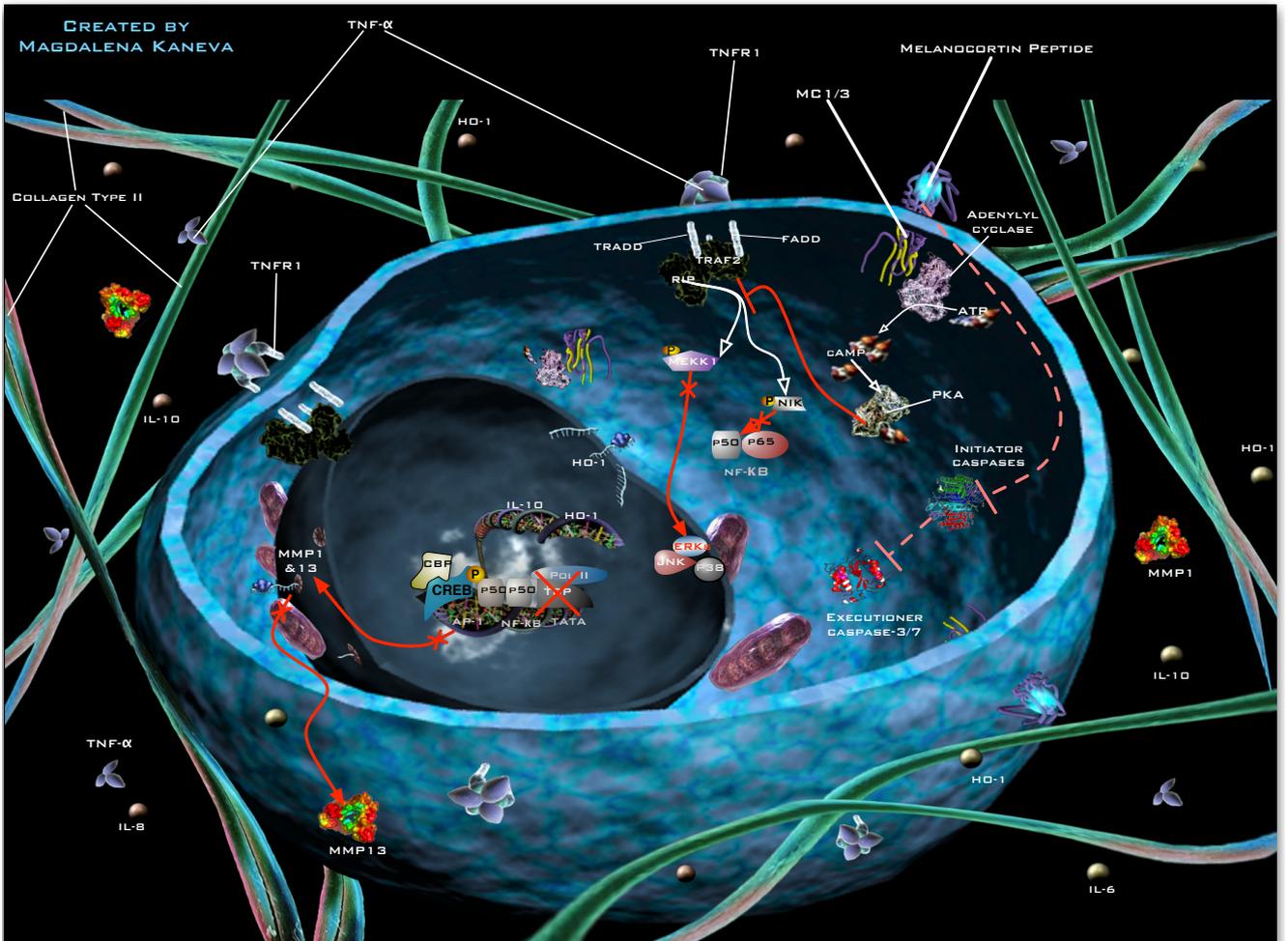
In the presence of TNF-α,
Mechanical Injury,
or change in ECM osmolarity

Chondrocytes express functional MCR 1 and 3
upon binding of melanocortin peptides to the MCR

IL-1β, IL-6,
IL-8, MCP-1,
iNOS,
Caspase-3/7,
apoptosis
MMP1, MMP13

Downstream Inhibition
Direct Stimulation
Direct Inhibition
Indirect Inhibition

261
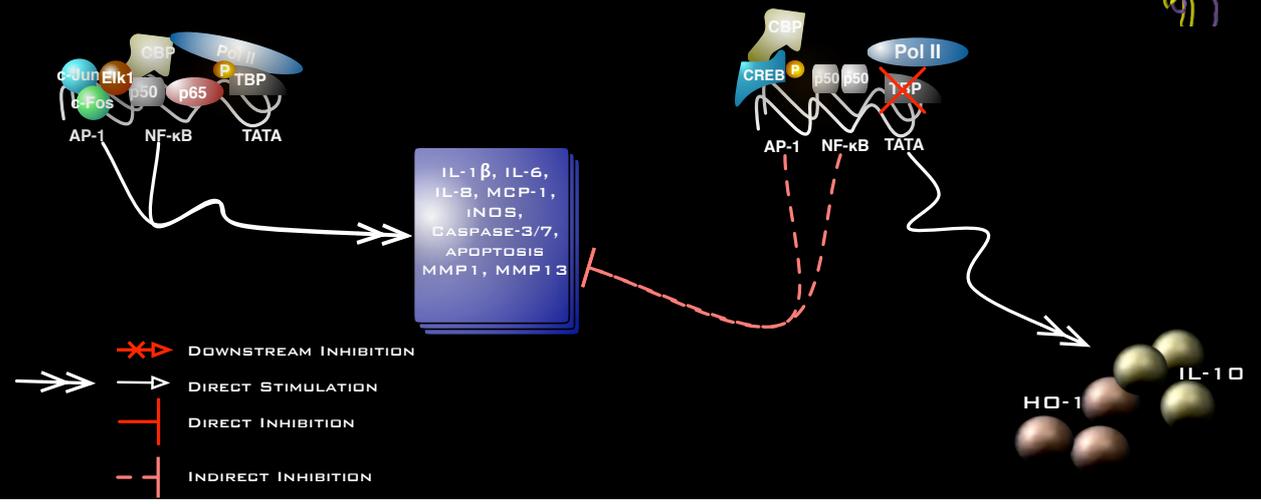
Figure 5.1 Melanocortins modulate pro-inflammatory cytokine production and exhert anti-infammatory and cytoprotective properties in chondrocytes.

The binding of melanocortins to either MC_1 or MC_3, functionally active on the membrane of chondrocytes, increases cAMP, which activates protein kinase A (PKA), therefore leading to four main effects. First, PKA activation induces the phosphorylation of the cAMP-responsive-element-binding protein (CREB) prevents the association of CBP with p65. b) The activated PKA hinders IκB kinase (IKK), which stabilizes the IκB inhibitor and stops nuclear translocation of NF-κB. c) PKA activation prevents MAPK/ERK kinase kinase 1 (MEKK1) phosphorylation and activation, and activation of p38 and TATA-binding protein (TBP). Non-phosphorylated TBP is unable to bind to the TATA box and to form dynamic trans-activating complex with CBP and NF-κB. A reduction in the amounts of nuclear p65, CBP and phosphorylated TBP inhibits the formation of the conformationally active trans-activating complex that is required for the transcription of most cytokine and chemokine genes, therefore a great reduction in the synthesis of IL-1β, IL-6, IL-8 and MCP-1 by the chondrocyte is observed. d) Fourth, inhibition of MEKK1 deactivates JUN kinase (JNK) and cJUN phosphorylation. The composition of the activator protein 1 (AP1) complex changes from the transcriptionally active cJun–cJun, to the transcriptionally inactive cJun–cFos or CREB. In addition, melanocortin peptides inhibit chondrocyte apoptosis by significantly inhibiting TNF-α-induced caspase-3 production and deactivating both executioner caspases - caspase-3 and caspase-7. Furthermore, activation of MC1 and MC3 led to significant up-regulation of production of IL-10 and HO-1, which support and assist the resolution of inflammation.

The final consequence is that the transcriptional machinery of chondrocytes is significantly disrupted by the melanocortin treatment, thereby driving pro-resolution of inflammation and emphasizing the anti-inflammatory and cytoprotective properties of the melanocortin peptides. This study exploits the melanocortin pathways as a first step towards possible development of future anti-inflammatory therapeutics and emphasized the need of further research in this direction.

5.2   Future work

- To investigate the effect of melanocortin peptides on collagen production by activated/osteoarthritic chondrocytes.

- To localize *IL-6*, *IL-8*, *MMP1* and *MMP13* gene loci to either euchromatin or heterochromatin in healthy and osteoarthritic chondrocytes and to investigate the effect of selective melanocortin peptides on the localization of these gene loci.

- To scrutinize the mechanism of hyper- and hypomethylation of *MMP1*, *MMP3* and *MMP13* following stimulated/osteoarthritic chondrocytes; to evaluate how epigenetic changes may relate to the pathogenesis of osteoarthritis.

- To investigate the effect of melanocortin treatment on cytokine and MMP genes methylation status in normal and osteoarthritic chondrocytes.

- To evaluate the peptides in pre-clinical models of OA and to look at the effect of these peptides on human tissue.

Chapter 6
References

ADACHI, S., NAKANO, T., VLIAGOFTIS, H. & METCALFE, D. D. 1999. Receptor-mediated modulation of murine mast cell function by alpha-melanocyte stimulating hormone. *J Immunol,* 163**,** 3363-8.

AHMED, S., RAHMAN, A., HASNAIN, A., GOLDBERG, V. M. & HAQQI, T. M. 2003. Phenyl N-tert-butylnitrone down-regulates interleukin-1 beta-stimulated matrix metalloproteinase-13 gene expression in human chondrocytes: suppression of c-Jun NH2-terminal kinase, p38-mitogen-activated protein kinase and activating protein-1. *J Pharmacol Exp Ther,* 305**,** 981-8.

AIGNER, T., HEMMEL, M., NEUREITER, D., GEBHARD, P. M., ZEILER, G., KIRCHNER, T. & MCKENNA, L. 2001. Apoptotic cell death is not a widespread phenomenon in normal aging and osteoarthritis human articular knee cartilage: a study of proliferation, programmed cell death (apoptosis), and viability of chondrocytes in normal and osteoarthritic human knee cartilage. *Arthritis Rheum,* 44**,** 1304-12.

AIGNER, T., KIM, H. A. & ROACH, H. I. 2004a. Apoptosis in osteoarthritis. *Rheum Dis Clin North Am,* 30**,** 639-53, xi.

AIGNER, T., ROSE, J., MARTIN, J. & BUCKWALTER, J. 2004b. Aging theories of primary osteoarthritis: from epidemiology to molecular biology. *Rejuvenation Res,* 7**,** 134-45.

AIGNER, T. & STOVE, J. 2003. Collagens--major component of the physiological cartilage matrix, major target of cartilage degeneration, major tool in cartilage repair. *Adv Drug Deliv Rev,* 55**,** 1569-93.

AIZAWA, T., KON, T., EINHORN, T. A. & GERSTENFELD, L. C. 2001. Induction of apoptosis in chondrocytes by tumor necrosis factor-alpha. *J Orthop Res,* 19**,** 785-96.

AJUEBOR, M. N., FLOWER, R. J., HANNON, R., CHRISTIE, M., BOWERS, K., VERITY, A. & PERRETTI, M. 1998. Endogenous monocyte chemoattractant protein-1 recruits monocytes in the zymosan peritonitis model. *J Leukoc Biol,* 63**,** 108-16.

AKAHOSHI, T., ENDO, H., KONDO, H., KASHIWAZAKI, S., KASAHARA, T., MUKAIDA, N., HARADA, A. & MATSUSHIMA, K. 1994. Essential involvement of interleukin-8 in neutrophil recruitment in rabbits with acute experimental arthritis induced by lipopolysaccharide and interleukin-1. *Lymphokine Cytokine Res,* 13**,** 113-6.

ALEKSEEV, E. I., SHAKHBAZIAN, I. E., ULYBINA, O. V. & KHUDOLEEVA, E. 2001. [Effectiveness and side-effects of long-term treatment with glucocorticoids in patients with juvenile rheumatoid arthritis]. *Klin Med (Mosk),* 79**,** 33-8.

ALLEN, K. D. 2010. Racial and ethnic disparities in osteoarthritis phenotypes. *Curr Opin Rheumatol,* 22**,** 528-32.

ALLEN, K. D., COFFMAN, C. J., GOLIGHTLY, Y. M., STECHUCHAK, K. M., VOILS, C. I. & KEEFE, F. J. 2010. Comparison of pain measures among patients with osteoarthritis. *J Pain,* 11**,** 522-7.

ALNEMRI, E. S., LIVINGSTON, D. J., NICHOLSON, D. W., SALVESEN, G., THORNBERRY, N. A., WONG, W. W. & YUAN, J. 1996. Human ICE/CED-3 protease nomenclature. *Cell,* 87**,** 171.

AMIN, A. R., ATTUR, M., PATEL, R. N., THAKKER, G. D., MARSHALL, P. J., REDISKE, J., STUCHIN, S. A., PATEL, I. R. & ABRAMSON, S. B. 1997. Superinduction of cyclooxygenase-2 activity in human osteoarthritis-affected cartilage. Influence of nitric oxide. *J Clin Invest,* 99**,** 1231-7.

ANDERSON, J. J. & FELSON, D. T. 1988. Factors associated with osteoarthritis of the knee in the first national Health and Nutrition Examination Survey (HANES I). Evidence for an association with overweight, race, and physical demands of work. *Am J Epidemiol,* 128**,** 179-89.

ARDEN, N. & NEVITT, M. C. 2006. Osteoarthritis: epidemiology. *Best Pract Res Clin Rheumatol,* 20**,** 3-25.

ATTUR, M., AL-MUSSAWIR, H. E., PATEL, J., KITAY, A., DAVE, M., PALMER, G., PILLINGER, M. H. & ABRAMSON, S. B. 2008. Prostaglandin E2 exerts catabolic effects in osteoarthritis cartilage: evidence for signaling via the EP4 receptor. *J Immunol,* 181**,** 5082-8.

ATTUR, M. G., DAVE, M., AKAMATSU, M., KATOH, M. & AMIN, A. R. 2002. Osteoarthritis or osteoarthrosis: the definition of inflammation becomes a semantic issue in the genomic era of molecular medicine. *Osteoarthritis Cartilage,* 10**,** 1-4.

ATTUR, M. G., DAVE, M., CIPOLLETTA, C., KANG, P., GOLDRING, M. B., PATEL, I. R., ABRAMSON, S. B. & AMIN, A. R. 2000. Reversal of autocrine and paracrine effects of interleukin 1 (IL-1) in human arthritis by type II IL-1 decoy receptor. Potential for pharmacological intervention. *J Biol Chem,* 275**,** 40307-15.

AUPHAN, N., DIDONATO, J. A., ROSETTE, C., HELMBERG, A. & KARIN, M. 1995. Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis. *Science,* 270**,** 286-90.

AURON, P. E., ROSENWASSER, L. J., MATSUSHIMA, K., COPELAND, T., DINARELLO, C. A., OPPENHEIM, J. J. & WEBB, A. C. 1985. Human and murine interleukin 1 possess sequence and structural similarities. *J Mol Cell Immunol,* 2**,** 169-77.

AVOUAC, J., GOSSEC, L. & DOUGADOS, M. 2006. Diagnostic and predictive value of anti-cyclic citrullinated protein antibodies in rheumatoid arthritis: a systematic literature review. *Ann Rheum Dis,* 65**,** 845-51.

BAGGIOLINI, M. 1998. Chemokines and leukocyte traffic. *Nature,* 392**,** 565-8.

BARACOS, V., RODEMANN, H. P., DINARELLO, C. A. & GOLDBERG, A. L. 1983. Stimulation of muscle protein degradation and prostaglandin E2 release by leukocytic pyrogen (interleukin-1). A mechanism for the increased degradation of muscle proteins during fever. *N Engl J Med,* 308**,** 553-8.

BARRETT, A. J. 1980. The many forms and functions of cellular proteinases. *Fed Proc,* 39**,** 9-14.

BARTHOLOMEW, B. A. 1984. Rapid and sensitive method for the assay of nitrate in urine using nitrate reductase enzyme of Escherichia coli. *Food Chem Tox,* 22**,** 541-543.

BAU, B., GEBHARD, P. M., HAAG, J., KNORR, T., BARTNIK, E. & AIGNER, T. 2002. Relative messenger RNA expression profiling of collagenases and aggrecanases in human articular chondrocytes in vivo and in vitro. *Arthritis Rheum,* 46**,** 2648-57.

BAUD, V., LIU, Z. G., BENNETT, B., SUZUKI, N., XIA, Y. & KARIN, M. 1999. Signaling by proinflammatory cytokines: oligomerization of TRAF2 and TRAF6 is sufficient for JNK and IKK activation and target gene induction via an amino-terminal effector domain. *Genes Dev,* 13**,** 1297-308.

BEASLEY, D. & COOPER, A. L. 1999. Constitutive expression of interleukin-1alpha precursor promotes human vascular smooth muscle cell proliferation. *Am J Physiol,* 276**,** H901-12.

BEATO, M., HERRLICH, P. & SCHUTZ, G. 1995. Steroid hormone receptors: many actors in search of a plot. *Cell,* 83**,** 851-7.

BELPERIO, J. A., KEANE, M. P., ARENBERG, D. A., ADDISON, C. L., EHLERT, J. E., BURDICK, M. D. & STRIETER, R. M. 2000. CXC chemokines in angiogenesis. *J Leukoc Biol,* 68**,** 1-8.

BENDER, S., HAUBECK, H. D., VAN DE LEUR, E., DUFHUES, G., SCHIEL, X., LAUWERIJNS, J., GREILING, H. & HEINRICH, P. C. 1990. Interleukin-1 beta induces synthesis and secretion of interleukin-6 in human chondrocytes. *FEBS Lett,* 263**,** 321-4.

BENJAMIN, M. & EVANS, E. J. 1990. Fibrocartilage. *J Anat,* 171**,** 1-15.

BENJAMIN, M. & RALPHS, J. R. 1998. Fibrocartilage in tendons and ligaments--an adaptation to compressive load. *J Anat,* 193 (Pt 4)**,** 481-94.

BENJANNET, S., RONDEAU, N., DAY, R., CHRETIEN, M. & SEIDAH, N. G. 1991. PC1 and PC2 are proprotein convertases capable of cleaving proopiomelanocortin at distinct pairs of basic residues. *Proc Natl Acad Sci U S A,* 88**,** 3564-8.

BERGSTROM, G., BJELLE, A., SORENSEN, L. B., SUNDH, V. & SVANBORG, A. 1986. Prevalence of rheumatoid arthritis, osteoarthritis, chondrocalcinosis and gouty arthritis at age 79. *J Rheumatol,* 13**,** 527-34.

BHARDWAJ, R., BECHER, E., MAHNKE, K., HARTMEYER, M., SCHWARZ, T., SCHOLZEN, T. & LUGER, T. A. 1997. Evidence for the differential expression of the functional alpha-melanocyte-stimulating hormone receptor MC-1 on human monocytes. *J Immunol,* 158**,** 3378-84.

BHARDWAJ, R. S., SCHWARZ, A., BECHER, E., MAHNKE, K., ARAGANE, Y., SCHWARZ, T. & LUGER, T. A. 1996. Pro-opiomelanocortin-derived peptides induce IL-10 production in human monocytes. *J Immunol,* 156**,** 2517-21.

BINGHAM, C. O., 3RD 2008. Emerging therapeutics for rheumatoid arthritis. *Bull NYU Hosp Jt Dis,* 66**,** 210-5.

BLANCO, F. J., GUITIAN, R., VAZQUEZ-MARTUL, E., DE TORO, F. J. & GALDO, F. 1998. Osteoarthritis chondrocytes die by apoptosis. A possible pathway for osteoarthritis pathology. *Arthritis Rheum,* 41**,** 284-9.

BOBACZ, K., SUNK, I. G., HOFSTAETTER, J. G., AMOYO, L., TOMA, C. D., AKIRA, S., WEICHHART, T., SAEMANN, M. & SMOLEN, J. S. 2007. Toll-like receptors and chondrocytes: the lipopolysaccharide-induced decrease in cartilage matrix synthesis is dependent on the presence of toll-like receptor 4 and antagonized by bone morphogenetic protein 7. *Arthritis Rheum,* 56**,** 1880-93.

BORDEN, P. & HELLER, R. A. 1997. Transcriptional control of matrix metalloproteinases and the tissue inhibitors of matrix metalloproteinases. *Crit Rev Eukaryot Gene Expr,* 7**,** 159-78.

BORRELLI, J., JR. & RICCI, W. M. 2004. Acute effects of cartilage impact. *Clin Orthop Relat Res***,** 33-9.

BORZI, R. M., MAZZETTI, I., MACOR, S., SILVESTRI, T., BASSI, A., CATTINI, L. & FACCHINI, A. 1999. Flow cytometric analysis of intracellular chemokines in chondrocytes in vivo: constitutive expression and enhancement in osteoarthritis and rheumatoid arthritis. *FEBS Lett,* 455**,** 238-42.

BOSTON, B. A. 1999. The role of melanocortins in adipocyte function. *Ann N Y Acad Sci,* 885**,** 75-84.

BOSTON, B. A. & CONE, R. D. 1996. Characterization of melanocortin receptor subtype expression in murine adipose tissues and in the 3T3-L1 cell line. *Endocrinology,* 137**,** 2043-50.

BRAY, G. A. 2004. Medical consequences of obesity. *J Clin Endocrinol Metab,* 89**,** 2583-9.

BRENNAN, F. M. & MCINNES, I. B. 2008. Evidence that cytokines play a role in rheumatoid arthritis. *J Clin Invest,* 118**,** 3537-45.

BRINCKERHOFF, C. E. 1992. Regulation of metalloproteinase gene expression: implications for osteoarthritis. *Crit Rev Eukaryot Gene Expr,* 2**,** 145-64.

BROSTJAN, C., ANRATHER, J., CSIZMADIA, V., STROKA, D., SOARES, M., BACH, F. H. & WINKLER, H. 1996. Glucocorticoid-mediated repression of NFkappaB activity in endothelial cells does not involve induction of IkappaBalpha synthesis. *J Biol Chem,* 271**,** 19612-6.

BRUNE, B., VON KNETHEN, A. & SANDAU, K. B. 1999. Nitric oxide (NO): an effector of apoptosis. *Cell Death Differ,* 6**,** 969-75.

BRZOSKA, T., KALDEN, D. H., SCHOLZEN, T. & LUGER, T. A. 1999. Molecular basis of the alpha-MSH/IL-1 antagonism. *Ann N Y Acad Sci,* 885**,** 230-8.

BUCKWALTER, J. A., COOPER, R. R. & MAYNARD, J. A. 1976. Elastic fibers in human intervertebral discs. *J Bone Joint Surg Am,* 58**,** 73-6.

BUCKWALTER, J. A. & MANKIN, H. J. 1998. Articular cartilage: degeneration and osteoarthritis, repair, regeneration, and transplantation. *Instr Course Lect,* 47**,** 487-504.

BUGGY, J. J. 1998. Binding of alpha-melanocyte-stimulating hormone to its G-protein-coupled receptor on B-lymphocytes activates the Jak/STAT pathway. *Biochem J,* 331 (Pt 1)**,** 211-6.

BUSH, P. G. & HALL, A. C. 2001a. The osmotic sensitivity of isolated and in situ bovine articular chondrocytes. *J Orthop Res,* 19**,** 768-78.

BUSH, P. G. & HALL, A. C. 2001b. Regulatory volume decrease (RVD) by isolated and in situ bovine articular chondrocytes. *J Cell Physiol,* 187**,** 304-14.

BUSH, P. G. & HALL, A. C. 2005. Passive osmotic properties of in situ human articular chondrocytes within non-degenerate and degenerate cartilage. *J Cell Physiol,* 204**,** 309-19.

BUSH, P. G., HODKINSON, P. D., HAMILTON, G. L. & HALL, A. C. 2005. Viability and volume of in situ bovine articular chondrocytes-changes following a single impact and effects of medium osmolarity. *Osteoarthritis Cartilage,* 13**,** 54-65.

BUTLER, A. A. & CONE, R. D. 2003. Knockout studies defining different roles for melanocortin receptors in energy homeostasis. *Ann N Y Acad Sci,* 994**,** 240-5.

CALDENHOVEN, E., LIDEN, J., WISSINK, S., VAN DE STOLPE, A., RAAIJMAKERS, J., KOENDERMAN, L., OKRET, S., GUSTAFSSON, J. A. & VAN DER SAAG, P. T. 1995. Negative cross-talk between RelA and the glucocorticoid receptor: a possible mechanism for the antiinflammatory action of glucocorticoids. *Mol Endocrinol,* 9**,** 401-12.

CAMPO, G. M., AVENOSO, A., CAMPO, S., D'ASCOLA, A., TRAINA, P., RUGOLO, C. A. & CALATRONI, A. 2010. Differential effect of molecular mass hyaluronan on lipopolysaccharide-induced damage in chondrocytes. *Innate Immun,* 16**,** 48-63.

CAMPO, G. M., AVENOSO, A., CAMPO, S., D'ASCOLA, A., TRAINA, P., SAMA, D. & CALATRONI, A. 2008. Purified human plasma glycosaminoglycans reduced NF-kappaB activation, pro-inflammatory cytokine production and apoptosis in LPS-treated chondrocytes. *Innate Immun,* 14**,** 233-46.

CANNON, C. P., CURTIS, S. P., FITZGERALD, G. A., KRUM, H., KAUR, A., BOLOGNESE, J. A., REICIN, A. S., BOMBARDIER, C., WEINBLATT, M. E., VAN DER HEIJDE, D., ERDMANN, E. & LAINE, L. 2006. Cardiovascular outcomes with etoricoxib and diclofenac in patients with osteoarthritis and rheumatoid arthritis in the Multinational Etoricoxib and Diclofenac Arthritis Long-term (MEDAL) programme: a randomised comparison. *Lancet,* 368**,** 1771-81.

CARMAN, C. V. & BENOVIC, J. L. 1998. G-protein-coupled receptors: turn-ons and turn-offs. *Curr Opin Neurobiol,* 8**,** 335-44.

CASTRO, M. G. & MORRISON, E. 1997. Post-translational processing of proopiomelanocortin in the pituitary and in the brain. *Crit Rev Neurobiol,* 11**,** 35-57.

CATANIA, A., COLOMBO, G., ROSSI, C., CARLIN, A., SORDI, A., LONATI, C., TURCATTI, F., LEONARDI, P., GRIECO, P. & GATTI, S. 2006. Antimicrobial properties of alpha-MSH and related synthetic melanocortins. *ScientificWorldJournal,* 6**,** 1241-6.

CATANIA, A., CUTULI, M., GAROFALO, L., AIRAGHI, L., VALENZA, F., LIPTON, J. M. & GATTINONI, L. 2000. Plasma concentrations and anti-L-cytokine effects of alpha-melanocyte stimulating hormone in septic patients. *Crit Care Med,* 28**,** 1403-7.

CATANIA, A., DELGADO, R., AIRAGHI, L., CUTULI, M., GAROFALO, L., CARLIN, A., DEMITRI, M. T. & LIPTON, J. M. 1999. alpha-MSH in systemic inflammation. Central and peripheral actions. *Ann N Y Acad Sci,* 885**,** 183-7.

CATANIA, A., GATTI, S., COLOMBO, G. & LIPTON, J. M. 2004. Targeting melanocortin receptors as a novel strategy to control inflammation. *Pharmacol Rev,* 56**,** 1-29.

CATANIA, A., GERLONI, V., PROCACCIA, S., AIRAGHI, L., MANFREDI, M. G., LOMATER, C., GROSSI, L. & LIPTON, J. M. 1994. The anticytokine neuropeptide alpha-melanocyte-stimulating hormone in synovial fluid of patients with rheumatic diseases: comparisons with other anticytokine molecules. *Neuroimmunomodulation,* 1**,** 321-8.

CATANIA, A., RAJORA, N., CAPSONI, F., MINONZIO, F., STAR, R. A. & LIPTON, J. M. 1996. The neuropeptide alpha-MSH has specific receptors on neutrophils and reduces chemotaxis in vitro. *Peptides,* 17**,** 675-9.

CAWSTON, T. E., MILNER, J. M., CATTERALL, J. B. & ROWAN, A. D. 2003. Cytokine synergy, collagenases and cartilage collagen breakdown. *Biochem Soc Symp***,** 125-33.

CAWSTON, T. E. & WILSON, A. J. 2006. Understanding the role of tissue degrading enzymes and their inhibitors in development and disease. *Best Pract Res Clin Rheumatol,* 20**,** 983-1002.

CERIANI, G., MACALUSO, A., CATANIA, A. & LIPTON, J. M. 1994. Central neurogenic antiinflammatory action of alpha-MSH: modulation of peripheral inflammation induced by cytokines and other mediators of inflammation. *Neuroendocrinology,* 59**,** 138-43.

CHAKRABORTI, S., MANDAL, M., DAS, S., MANDAL, A. & CHAKRABORTI, T. 2003. Regulation of matrix metalloproteinases: an overview. *Mol Cell Biochem,* 253**,** 269-85.

CHEN, W., KELLY, M. A., OPITZ-ARAYA, X., THOMAS, R. E., LOW, M. J. & CONE, R. D. 1997. Exocrine gland dysfunction in MC5-R-deficient mice: evidence for coordinated regulation of exocrine gland function by melanocortin peptides. *Cell,* 91**,** 789-98.

CHEN, Y. F., JOBANPUTRA, P., BARTON, P., BRYAN, S., FRY-SMITH, A., HARRIS, G. & TAYLOR, R. S. 2008. Cyclooxygenase-2 selective non-steroidal anti-inflammatory drugs (etodolac, meloxicam, celecoxib, rofecoxib, etoricoxib, valdecoxib and lumiracoxib) for osteoarthritis and

rheumatoid arthritis: a systematic review and economic evaluation. *Health Technol Assess,* 12**,** 1-278, iii.

CHENG, Y., WANG, M., YU, Y., LAWSON, J., FUNK, C. D. & FITZGERALD, G. A. 2006. Cyclooxygenases, microsomal prostaglandin E synthase-1, and cardiovascular function. *J Clin Invest,* 116**,** 1391-9.

CHHAJLANI, V. 1996. Distribution of cDNA for melanocortin receptor subtypes in human tissues. *Biochem Mol Biol Int,* 38**,** 73-80.

CHHAJLANI, V. & WIKBERG, J. E. 1992. Molecular cloning and expression of the human melanocyte stimulating hormone receptor cDNA. *FEBS Lett,* 309**,** 417-20.

CHILAPPA, C. S., ARONOW, W. S., SHAPIRO, D., SPERBER, K., PATEL, U. & ASH, J. Y. 2010. Gout and hyperuricemia. *Compr Ther,* 36**,** 3-13.

CHOI, Y. H., BURDICK, M. D. & STRIETER, R. M. 2009. Human circulating fibrocytes have the capacity to differentiate osteoblasts and chondrocytes. *Int J Biochem Cell Biol,* 42**,** 662-71.

CHU, C. Q., FIELD, M., FELDMANN, M. & MAINI, R. N. 1991. Localization of tumor necrosis factor alpha in synovial tissues and at the cartilage-pannus junction in patients with rheumatoid arthritis. *Arthritis Rheum,* 34**,** 1125-32.

CLAASSEN, H., SCHICHT, M. & PAULSEN, F. 2011. Impact of sex hormones, insulin, growth factors and peptides on cartilage health and disease. *Prog Histochem Cytochem,* 45**,** 239-93.

CLAASSEN, H., STEFFEN, R., HASSENPFLUG, J., VAROGA, D., WRUCK, C. J., BRANDENBURG, L. O. & PUFE, T. 2010. 17beta-estradiol reduces expression of MMP-1, -3, and -13 in human primary articular chondrocytes from female patients cultured in a three dimensional alginate system. *Cell Tissue Res,* 342**,** 283-93.

CLARK, A. J., MCLOUGHLIN, L. & GROSSMAN, A. 1993. Familial glucocorticoid deficiency associated with point mutation in the adrenocorticotropin receptor. *Lancet,* 341**,** 461-2.

CLARK, R. B. 1986. Receptor desensitization. *Adv Cyclic Nucl Prot Phos Res,* 20**,** 151 - 209.

COLE, A. G. & HALL, B. K. 2004. The nature and significance of invertebrate cartilages revisited: distribution and histology of cartilage and cartilage-like tissues within the Metazoa. *Zoology (Jena),* 107**,** 261-73.

CRONSTEIN, B. N. & TERKELTAUB, R. 2006. The inflammatory process of gout and its treatment. *Arthritis Res Ther,* 8 Suppl 1**,** S3.

CROXTALL, J. D. & FLOWER, R. J. 1992. Lipocortin 1 mediates dexamethasone-induced growth arrest of the A549 lung adenocarcinoma cell line. *Proc Natl Acad Sci U S A,* 89**,** 3571-5.

D'AGOSTINO, G. & DIANO, S. 2010. Alpha-melanocyte stimulating hormone: production and degradation. *J Mol Med,* 88**,** 1195-201.

D'LIMA, D. D., HASHIMOTO, S., CHEN, P. C., COLWELL, C. W., JR. & LOTZ, M. K. 2001a. Impact of mechanical trauma on matrix and cells. *Clin Orthop Relat Res,* S90-9.

D'LIMA, D. D., HASHIMOTO, S., CHEN, P. C., LOTZ, M. K. & COLWELL, C. W., JR. 2001b. In vitro and in vivo models of cartilage injury. *J Bone Joint Surg Am,* 83-A Suppl 2**,** 22-4.

DAHAGHIN, S., TEHRANI-BANIHASHEMI, S. A., FAEZI, S. T., JAMSHIDI, A. R. & DAVATCHI, F. 2009. Squatting, sitting on the floor, or cycling: are life-long daily activities risk factors for clinical knee osteoarthritis? Stage III results of a community-based study. *Arthritis Rheum,* 61**,** 1337-42.

DANIELSSON, L., LINDBERG, H. & NILSSON, B. 1984. Prevalence of coxarthrosis. *Clin Orthop Relat Res***,** 110-5.

DAVID, F., FARLEY, J., HUANG, H., LAVOIE, J. P. & LAVERTY, S. 2007. Cytokine and chemokine gene expression of IL-1beta stimulated equine articular chondrocytes. *Vet Surg,* 36**,** 221-7.

DAVIES, T. H., NING, Y. M. & SANCHEZ, E. R. 2005. Differential control of glucocorticoid receptor hormone-binding function by tetratricopeptide repeat (TPR) proteins and the immunosuppressive ligand FK506. *Biochemistry,* 44**,** 2030-8.

DAYER, J. M., BREARD, J., CHESS, L. & KRANE, S. M. 1979. Participation of monocyte-macrophages and lymphocytes in the production of a factor that stimulates collagenase and prostaglandin release by rheumatoid synovial cells. *J Clin Invest,* 64**,** 1386-92.

DE BOER, R. A., VOORS, A. A., MUNTENDAM, P., VAN GILST, W. H. & VAN VELDHUISEN, D. J. 2009. Galectin-3: a novel mediator of heart failure development and progression. *Eur J Heart Fail,* 11**,** 811-7.

DEFORGE, L. E., KENNEY, J. S., JONES, M. L., WARREN, J. S. & REMICK, D. G. 1992. Biphasic production of IL-8 in lipopolysaccharide (LPS)-stimulated human whole blood. Separation of LPS- and cytokine-stimulated components using anti-tumor necrosis factor and anti-IL-1 antibodies. *J Immunol,* 148**,** 2133-41.

DEL CARLO, M., JR. & LOESER, R. F. 2002. Nitric oxide-mediated chondrocyte cell death requires the generation of additional reactive oxygen species. *Arthritis Rheum,* 46**,** 394-403.

DELGADO HERNANDEZ, R., DEMITRI, M. T., CARLIN, A., MEAZZA, C., VILLA, P., GHEZZI, P., LIPTON, J. M. & CATANIA, A. 1999. Inhibition of systemic inflammation by central action of the neuropeptide alpha-melanocyte-stimulating hormone. *Neuroimmunomodulation,* 6**,** 187-92.

DENISOV, L. N., NASONOVA, V. A., KORESHKOV, G. G. & KASHEVAROVA, N. G. 2010. [Role of obesity in the development of osteoarthrosis and concomitant diseases]. *Ter Arkh,* 82**,** 34-7.

DI GIOVINE, F. S., MALAWISTA, S. E., THORNTON, E. & DUFF, G. W. 1991. Urate crystals stimulate production of tumor necrosis factor alpha from human blood monocytes and synovial cells. Cytokine mRNA and protein kinetics, and cellular distribution. *J Clin Invest,* 87**,** 1375-81.

DIAKONOVA, M., GERKE, V., ERNST, J., LIAUTARD, J. P., VAN DER VUSSE, G. & GRIFFITHS, G. 1997. Localization of five annexins in J774

macrophages and on isolated phagosomes. *J Cell Sci,* 110 (Pt 10)**,** 1199-213.

DIBATTISTA, J. A., DORE, S., MORIN, N. & ABRIBAT, T. 1996. Prostaglandin E2 up-regulates insulin-like growth factor binding protein-3 expression and synthesis in human articular chondrocytes by a c-AMP-independent pathway: role of calcium and protein kinase A and C. *J Cell Biochem,* 63**,** 320-33.

DICKINSON, D. P. 2002. Cysteine peptidases of mammals: their biological roles and potential effects in the oral cavity and other tissues in health and disease. *Crit Rev Oral Biol Med,* 13**,** 238-75.

DIEPPE, P. & KIRWAN, J. 1994. The localization of osteoarthritis. *Br J Rheumatol,* 33**,** 201-3.

DING, H., SOLOVIEVA, S., VEHMAS, T., TAKALA, E. P. & LEINO-ARJAS, P. 2010. Hand osteoarthritis and pinch grip strength among middle-aged female dentists and teachers. *Scand J Rheumatol,* 39**,** 84-7.

DIXON, W. G., HYRICH, K. L., WATSON, K. D., LUNT, M., GALLOWAY, J., USTIANOWSKI, A. & SYMMONS, D. P. 2010. Drug-specific risk of tuberculosis in patients with rheumatoid arthritis treated with anti-TNF therapy: results from the British Society for Rheumatology Biologics Register (BSRBR). *Ann Rheum Dis,* 69**,** 522-8.

DORSCHEID, D. R., PATCHELL, B. J., ESTRADA, O., MARROQUIN, B., TSE, R. & WHITE, S. R. 2006. Effects of corticosteroid-induced apoptosis on airway epithelial wound closure in vitro. *Am J Physiol Lung Cell Mol Physiol,* 291**,** L794-801.

DOSSUMBEKOVA, A., ANGHELINA, M., MADHAVAN, S., HE, L., QUAN, N., KNOBLOCH, T. & AGARWAL, S. 2007. Biomechanical signals inhibit IKK activity to attenuate NF-kappaB transcription activity in inflamed chondrocytes. *Arthritis Rheum,* 56**,** 3284-96.

DROUIN, J. & GOODMAN, H. M. 1980. Most of the coding region of rat ACTH beta--LPH precursor gene lacks intervening sequences. *Nature,* 288**,** 610-3.

DUMIC, J., DABELIC, S. & FLOGEL, M. 2006. Galectin-3: an open-ended story. *Biochim Biophys Acta,* 1760**,** 616-35.

ERTEL, W., KEEL, M., BONACCIO, M., STECKHOLZER, U., GALLATI, H., KENNEY, J. S. & TRENTZ, O. 1995. Release of anti-inflammatory mediators after mechanical trauma correlates with severity of injury and clinical outcome. *J Trauma,* 39**,** 879-85; discussion 885-7.

ETTINGER, W. H., JR. & AFABLE, R. F. 1994. Physical disability from knee osteoarthritis: the role of exercise as an intervention. *Med Sci Sports Exerc,* 26**,** 1435-40.

EVANS, J. F., NIU, Q. T., CANAS, J. A., SHEN, C. L., ALOIA, J. F. & YEH, J. K. 2004. ACTH enhances chondrogenesis in multipotential progenitor cells and matrix production in chondrocytes. *Bone,* 35**,** 96-107.

EVANS, J. F., SHEN, C. L., POLLACK, S., ALOIA, J. F. & YEH, J. K. 2005. Adrenocorticotropin evokes transient elevations in intracellular free calcium ([Ca2+]i) and increases basal [Ca2+]i in resting chondrocytes through a phospholipase C-dependent mechanism. *Endocrinology,* 146, 3123-32.

EYRE, D. R. 2004. Collagens and cartilage matrix homeostasis. *Clin Orthop Relat Res,* S118-22.

EYRE, D. R. & MUIR, H. 1975. The distribution of different molecular species of collagen in fibrous, elastic and hyaline cartilages of the pig. *Biochem J,* 151, 595-602.

EYRE, D. R., WU, J. J. & WOODS, P. 1992. Cartilage-specific collagens: structural studies. *In:* KUETTNER KE, S. R., PEYRON JG, HASKELL VC (ed.) *Articular Cartilage and Osteoarthritis.* New York: Raven Press.

FAN, W., BOSTON, B. A., KESTERSON, R. A., HRUBY, V. J. & CONE, R. D. 1997. Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. *Nature,* 385, 165-8.

FAN, Z., BAU, B., YANG, H., SOEDER, S. & AIGNER, T. 2005. Freshly isolated osteoarthritic chondrocytes are catabolically more active than normal chondrocytes, but less responsive to catabolic stimulation with interleukin-1beta. *Arthritis Rheum,* 52, 136-43.

FELSON DT, LAWRENCE RC, DIEPPE PA & R, H. 2000. Osteoarthritis: New insights. Part 1: The disease and its risk factors. *Ann Intern Med,* 133, 635–646.

FELSON, D. T., NAIMARK, A., ANDERSON, J., KAZIS, L., CASTELLI, W. & MEENAN, R. F. 1987. The prevalence of knee osteoarthritis in the elderly. The Framingham Osteoarthritis Study. *Arthritis Rheum,* 30, 914-8.

FERGUSON, S. S., ZHANG, J., BARAK, L. S. & CARON, M. G. 1998. Molecular mechanisms of G protein-coupled receptor desensitization and resensitization. *Life Sci,* 62, 1561-5.

FERNANDES, J., TARDIF, G., MARTEL-PELLETIER, J., LASCAU-COMAN, V., DUPUIS, M., MOLDOVAN, F., SHEPPARD, M., KRISHNAN, B. R. & PELLETIER, J. P. 1999. In vivo transfer of interleukin-1 receptor antagonist gene in osteoarthritic rabbit knee joints: prevention of osteoarthritis progression. *Am J Pathol,* 154, 1159-69.

FERNANDES, J. C., MARTEL-PELLETIER, J. & PELLETIER, J. P. 2002. The role of cytokines in osteoarthritis pathophysiology. *Biorheology,* 39, 237-46.

FERNANDEZ, P., GUILLEN, M. I., GOMAR, F. & ALCARAZ, M. J. 2003. Expression of heme oxygenase-1 and regulation by cytokines in human osteoarthritic chondrocytes. *Biochem Pharmacol,* 66, 2049-52.

FINGER, F., SCHORLE, C., SODER, S., ZIEN, A., GOLDRING, M. B. & AIGNER, T. 2004. Phenotypic characterization of human chondrocyte cell line C-20/A4: a comparison between monolayer and alginate suspension culture. *Cells Tissues Organs,* 178, 65-77.

FIORINO, G., ALLEZ, M., MALESCI, A. & DANESE, S. 2009. Review article: anti TNF-alpha induced psoriasis in patients with inflammatory bowel disease. *Aliment Pharmacol Ther,* 29**,** 921-7.

FISCHER, P. & HILFIKER-KLEINER, D. 2007. Survival pathways in hypertrophy and heart failure: the gp130-STAT3 axis. *Basic Res Cardiol,* 102**,** 279-97.

FLOWER, R. J. & BLACKWELL, G. J. 1979. Anti-inflammatory steroids induce biosynthesis of a phospholipase A2 inhibitor which prevents prostaglandin generation. *Nature,* 278**,** 456-9.

FLOWER, R. J. & ROTHWELL, N. J. 1994. Lipocortin-1: cellular mechanisms and clinical relevance. *Trends Pharmacol Sci,* 15**,** 71-6.

FLOWER, R. J. & VANE, J. R. 1972. Inhibition of prostaglandin synthetase in brain explains the anti-pyretic activity of paracetamol (4-acetamidophenol). *Nature,* 240**,** 410-1.

FOKTER, S. K. & REPSE-FOKTER, A. 2010. Acute gouty arthritis in a patient after total knee arthroplasty. *Wien Klin Wochenschr,* 122**,** 366-7.

FONTANA, L., NEEL, S., CLAISE, J. M., UGHETTO, S. & CATILINA, P. 2007. Osteoarthritis of the thumb carpometacarpal joint in women and occupational risk factors: a case-control study. *J Hand Surg Am,* 32**,** 459-65.

FRANKLIN, J., INGVARSSON, T., ENGLUND, M. & LOHMANDER, S. 2010. Association between occupation and knee and hip replacement due to osteoarthritis: a case-control study. *Arthritis Res Ther,* 12**,** R102.

FRASER, A., FEARON, U., BILLINGHURST, R. C., IONESCU, M., REECE, R., BARWICK, T., EMERY, P., POOLE, A. R. & VEALE, D. J. 2003. Turnover of type II collagen and aggrecan in cartilage matrix at the onset of inflammatory arthritis in humans: relationship to mediators of systemic and local inflammation. *Arthritis Rheum,* 48**,** 3085-95.

FRIEDLANDER, R. M., GAGLIARDINI, V., ROTELLO, R. J. & YUAN, J. 1996. Functional role of interleukin 1 beta (IL-1 beta) in IL-1 beta-converting enzyme-mediated apoptosis. *J Exp Med,* 184**,** 717-24.

FROST, J. A., ALBERTS, A. S., SONTAG, E., GUAN, K., MUMBY, M. C. & FERAMISCO, J. R. 1994. Simian virus 40 small t antigen cooperates with mitogen-activated kinases to stimulate AP-1 activity. *Mol Cell Biol,* 14**,** 6244-52.

GADHER, S. J., EYRE, D. R., WOTTON, S. F., SCHMID, T. M. & WOOLLEY, D. E. 1990. Degradation of cartilage collagens type II, IX, X and XI by enzymes derived from human articular chondrocytes. *Matrix,* 10**,** 154-63.

GANTZ, I. & FONG, T. M. 2003. The melanocortin system. *Am J Physiol Endocrinol Metab,* 284**,** E468-74.

GANTZ, I., KONDA, Y., TASHIRO, T., SHIMOTO, Y., MIWA, H., MUNZERT, G., WATSON, S. J., DELVALLE, J. & YAMADA, T. 1993a. Molecular cloning of a novel melanocortin receptor. *J Biol Chem,* 268**,** 8246-50.

GANTZ, I., MIWA, H., KONDA, Y., SHIMOTO, Y., TASHIRO, T., WATSON, S. J., DELVALLE, J. & YAMADA, T. 1993b. Molecular cloning, expression, and

gene localization of a fourth melanocortin receptor. *J Biol Chem,* 268**,** 15174-9.

GANTZ, I., SHIMOTO, Y., KONDA, Y., MIWA, H., DICKINSON, C. J. & YAMADA, T. 1994. Molecular cloning, expression, and characterization of a fifth melanocortin receptor. *Biochem Biophys Res Commun,* 200**,** 1214-20.

GANTZ, I., TASHIRO, T., BARCROFT, C., KONDA, Y., SHIMOTO, Y., MIWA, H., GLOVER, T., MUNZERT, G. & YAMADA, T. 1993c. Localization of the genes encoding the melanocortin-2 (adrenocorticotropic hormone) and melanocortin-3 receptors to chromosomes 18p11.2 and 20q13.2-q13.3 by fluorescence in situ hybridization. *Genomics,* 18**,** 166-7.

GARCIA-CALVO, M., PETERSON, E. P., RASPER, D. M., VAILLANCOURT, J. P., ZAMBONI, R., NICHOLSON, D. W. & THORNBERRY, N. A. 1999. Purification and catalytic properties of human caspase family members. *Cell Death Differ,* 6**,** 362-9.

GARDNER, D. L. 1992. *The pathological Basis of Connective Tissue Disease.,* London, Edward Arnold.

GARRINGTON, T. P. & JOHNSON, G. L. 1999. Organization and regulation of mitogen-activated protein kinase signaling pathways. *Curr Opin Cell Biol,* 11**,** 211-8.

GATTI, S., COLOMBO, G., BUFFA, R., TURCATTI, F., GAROFALO, L., CARBONI, N., FERLA, L., FASSATI, L. R., LIPTON, J. M. & CATANIA, A. 2002. alpha-Melanocyte-stimulating hormone protects the allograft in experimental heart transplantation. *Transplantation,* 74**,** 1678-84.

GERKE, V. & MOSS, S. E. 2002. Annexins: from structure to function. *Physiol Rev,* 82**,** 331-71.

GETTING, S. J. 2002. Melanocortin peptides and their receptors: new targets for anti-inflammatory therapy. *Trends Pharmacol Sci,* 23**,** 447-9.

GETTING, S. J. 2006. Targeting melanocortin receptors as potential novel therapeutics. *Pharmacol Ther,* 111**,** 1-15.

GETTING, S. J., ALLCOCK, G. H., FLOWER, R. & PERRETTI, M. 2001. Natural and synthetic agonists of the melanocortin receptor type 3 possess anti-inflammatory properties. *J Leukoc Biol,* 69**,** 98-104.

GETTING, S. J., CHRISTIAN, H. C., FLOWER, R. J. & PERRETTI, M. 2002. Activation of melanocortin type 3 receptor as a molecular mechanism for adrenocorticotropic hormone efficacy in gouty arthritis. *Arthritis Rheum,* 46**,** 2765-75.

GETTING, S. J., CHRISTIAN, H. C., LAM, C. W., GAVINS, F. N., FLOWER, R. J., SCHIOTH, H. B. & PERRETTI, M. 2003. Redundancy of a functional melanocortin 1 receptor in the anti-inflammatory actions of melanocortin peptides: studies in the recessive yellow (e/e) mouse suggest an important role for melanocortin 3 receptor. *J Immunol,* 170**,** 3323-30.

GETTING, S. J., DI FILIPPO, C., CHRISTIAN, H. C., LAM, C. W., ROSSI, F., D'AMICO, M. & PERRETTI, M. 2004. MC-3 receptor and the inflammatory

mechanisms activated in acute myocardial infarct. *J Leukoc Biol,* 76**,** 845-53.

GETTING, S. J., GIBBS, L., CLARK, A. J., FLOWER, R. J. & PERRETTI, M. 1999. POMC gene-derived peptides activate melanocortin type 3 receptor on murine macrophages, suppress cytokine release, and inhibit neutrophil migration in acute experimental inflammation. *J Immunol,* 162**,** 7446-53.

GETTING, S. J., KANEVA, M., BHADRESA, Y., RENSHAW, D., LEONI, G., PATEL, H. B., KERRIGAN, P. M. & LOCKE, I. C. 2009. Melanocortin peptide therapy for the treatment of arthritic pathologies. *ScientificWorldJournal,* 9**,** 1394-414.

GETTING, S. J., LAM, C. W., CHEN, A. S., GRIECO, P. & PERRETTI, M. 2006. Melanocortin 3 receptors control crystal-induced inflammation. *FASEB J,* 20**,** 2234-41.

GETTING, S. J. & PERRETTI, M. 2000. MC3-R as a novel target for antiinflammatory therapy. *Drug News Perspect,* 13**,** 19-27.

GETTING, S. J. & PERRETTI, M. 2001. Arthritic diseases: melanocortin type 3 receptor agonists as potential therapeutics. *Curr Opin Investig Drugs,* 2**,** 1064-9.

GETTING, S. J., RIFFO-VASQUEZ, Y., PITCHFORD, S., KANEVA, M., GRIECO, P., PAGE, C. P., PERRETTI, M. & SPINA, D. 2008. A role for MC3R in modulating lung inflammation. *Pulm Pharmacol Ther,* 21**,** 866-73.

GILLARD, G. C., REILLY, H. C., BELL-BOOTH, P. G. & FLINT, M. H. 1979. The influence of mechanical forces on the glycosaminoglycan content of the rabbit flexor digitorum profundus tendon. *Connect Tissue Res,* 7**,** 37-46.

GOLDRING, M. B. 2000a. Osteoarthritis and cartilage: the role of cytokines. *Curr Rheumatol Rep,* 2**,** 459-65.

GOLDRING, M. B. 2000b. The role of the chondrocyte in osteoarthritis. *Arthritis Rheum,* 43**,** 1916-26.

GOLDRING, M. B. 2004. Immortalization of human articular chondrocytes for generation of stable, differentiated cell lines. *Methods Mol Med,* 100**,** 23-36.

GOLDRING, M. B. & BERENBAUM, F. 2004. The regulation of chondrocyte function by proinflammatory mediators: prostaglandins and nitric oxide. *Clin Orthop Relat Res***,** S37-46.

GOLDRING, M. B., BIRKHEAD, J. R., SUEN, L. F., YAMIN, R., MIZUNO, S., GLOWACKI, J., ARBISER, J. L. & APPERLEY, J. F. 1994a. Interleukin-1 beta-modulated gene expression in immortalized human chondrocytes. *J Clin Invest,* 94**,** 2307-16.

GOLDRING, M. B., FUKUO, K., BIRKHEAD, J. R., DUDEK, E. & SANDELL, L. J. 1994b. Transcriptional suppression by interleukin-1 and interferon-gamma of type II collagen gene expression in human chondrocytes. *J Cell Biochem,* 54**,** 85-99.

GOLDRING, M. B. & GOLDRING, S. R. 2007. Osteoarthritis. *J Cell Physiol,* 213**,** 626-34.

GOLDRING, M. B., OTERO, M., PLUMB, D. A., DRAGOMIR, C., FAVERO, M., EL HACHEM, K., HASHIMOTO, K., ROACH, H. I., OLIVOTTO, E., BORZI, R. M. & MARCU, K. B. 2011. Roles of inflammatory and anabolic cytokines in cartilage metabolism: signals and multiple effectors converge upon MMP-13 regulation in osteoarthritis. *Eur Cell Mater,* 21**,** 202-20.

GOLDRING, M. B., OTERO, M., TSUCHIMOCHI, K., IJIRI, K. & LI, Y. 2008. Defining the roles of inflammatory and anabolic cytokines in cartilage metabolism. *Ann Rheum Dis,* 67 Suppl 3**,** iii75-82.

GONZALEZ-REY, E., CHORNY, A. & DELGADO, M. 2007. Regulation of immune tolerance by anti-inflammatory neuropeptides. *Nat Rev Immunol,* 7**,** 52-63.

GOSSET, M., PIGENET, A., SALVAT, C., BERENBAUM, F. & JACQUES, C. 2010. Inhibition of matrix metalloproteinase-3 and -13 synthesis induced by IL-1beta in chondrocytes from mice lacking microsomal prostaglandin E synthase-1. *J Immunol,* 185**,** 6244-52.

GRABBE, S., BHARDWAJ, R. S., MAHNKE, K., SIMON, M. M., SCHWARZ, T. & LUGER, T. A. 1996. alpha-Melanocyte-stimulating hormone induces hapten-specific tolerance in mice. *J Immunol,* 156**,** 473-8.

GRABOWSKI, P. S., WRIGHT, P. K., VAN 'T HOF, R. J., HELFRICH, M. H., OHSHIMA, H. & RALSTON, S. H. 1997. Immunolocalization of inducible nitric oxide synthase in synovium and cartilage in rheumatoid arthritis and osteoarthritis. *Br J Rheumatol,* 36**,** 651-5.

GRANGE, L., NGUYEN, M. V., LARDY, B., DEROUAZI, M., CAMPION, Y., TROCME, C., PACLET, M. H., GAUDIN, P. & MOREL, F. 2006. NAD(P)H oxidase activity of Nox4 in chondrocytes is both inducible and involved in collagenase expression. *Antioxid Redox Signal,* 8**,** 1485-96.

GRASSEL, S., OPOLKA, A., ANDERS, S., STRAUB, R. H., GRIFKA, J., LUGER, T. A. & BOHM, M. 2009. The melanocortin system in articular chondrocytes: melanocortin receptors, pro-opiomelanocortin, precursor proteases, and a regulatory effect of alpha-melanocyte-stimulating hormone on proinflammatory cytokines and extracellular matrix components. *Arthritis Rheum,* 60**,** 3017-27.

GRIECO, P., BALSE, P. M., WEINBERG, D., MACNEIL, T. & HRUBY, V. J. 2000. D-Amino acid scan of gamma-melanocyte-stimulating hormone: importance of Trp(8) on human MC3 receptor selectivity. *J Med Chem,* 43**,** 4998-5002.

GRIECO, P., HAN, G., WEINBERG, D., VAN DER PLOEG, L. H. & HRUBY, V. J. 2002. Design and synthesis of highly potent and selective melanotropin analogues of SHU9119 modified at position 6. *Biochem Biophys Res Commun,* 292**,** 1075-80.

GRIFFON, N., MIGNON, V., FACCHINETTI, P., DIAZ, J., SCHWARTZ, J. C. & SOKOLOFF, P. 1994. Molecular cloning and characterization of the rat fifth melanocortin receptor. *Biochem Biophys Res Commun,* 200**,** 1007-14.

GUARINI, S., SCHIOTH, H. B., MIONI, C., CAINAZZO, M., FERRAZZA, G., GIULIANI, D., WIKBERG, J. E., BERTOLINI, A. & BAZZANI, C. 2002. MC(3) receptors are involved in the protective effect of melanocortins in myocardial ischemia/reperfusion-induced arrhythmias. *Naunyn Schmiedebergs Arch Pharmacol,* 366**,** 177-82.

GUAYDIER-SOUQUIERES, G., KOTZKI, P. O., SABATIER, J. P., BASSE-CATHALINAT, B. & LOEB, G. 1996. In corticosteroid-treated respiratory diseases, monofluorophosphate increases lumbar bone density: a double-masked randomized study. *Osteoporos Int,* 6**,** 171-7.

GUCCIONE, A. A. 1997. Osteoarthritis, comorbidity, and physical disability. *Baltimore, MD: The Johns Hopkins University Press***,** 84-98.

GUILAK, F., MEYER, B. C., RATCLIFFE, A. & MOW, V. C. 1994. The effects of matrix compression on proteoglycan metabolism in articular cartilage explants. *Osteoarthritis Cartilage,* 2**,** 91-101.

GUPTA, D., BEHERA, D., LALRINMAWIA, H. & DASH, R. J. 2000. Hypothalamo-pituitary-adrenal axis function in asthmatics taking low dose inhaled beclomethasone dipropionate. *J Assoc Physicians India,* 48**,** 682-4.

GUTMAN, A. B. & YU, T. F. 1950. Effects of adrenocorticotropic hormone (ACTH) in gout. *Am J Med,* 9**,** 24-30.

HADLEY, M. E., HRUBY, V. J., BLANCHARD, J., DORR, R. T., LEVINE, N., DAWSON, B. V., AL-OBEIDI, F. & SAWYER, T. K. 1998. Discovery and development of novel melanogenic drugs. Melanotan-I and -II. *Pharm Biotechnol,* 11**,** 575-95.

HAGLUND, L., BERNIER, S. M., ONNERFJORD, P. & RECKLIES, A. D. 2008. Proteomic analysis of the LPS-induced stress response in rat chondrocytes reveals induction of innate immune response components in articular cartilage. *Matrix Biol,* 27**,** 107-18.

HALL, A. C., HORWITZ, E. R. & WILKINS, R. J. 1996. The cellular physiology of articular cartilage. *Exp Physiol,* 81**,** 535-45.

HALL, A. G. 1998. Physiology of Cartilage. . *Hughes SaMID, editor: W. B. Saunders.***,** 308 p.

HALL, M. C., YOUNG, D. A., WATERS, J. G., ROWAN, A. D., CHANTRY, A., EDWARDS, D. R. & CLARK, I. M. 2003. The comparative role of activator protein 1 and Smad factors in the regulation of Timp-1 and MMP-1 gene expression by transforming growth factor-beta 1. *J Biol Chem,* 278**,** 10304-13.

HANNON, R., CROXTALL, J. D., GETTING, S. J., ROVIEZZO, F., YONA, S., PAUL-CLARK, M. J., GAVINS, F. N., PERRETTI, M., MORRIS, J. F., BUCKINGHAM, J. C. & FLOWER, R. J. 2003. Aberrant inflammation and resistance to glucocorticoids in annexin 1-/- mouse. *Faseb J,* 17**,** 253-5.

HARADA, A., SEKIDO, N., AKAHOSHI, T., WADA, T., MUKAIDA, N. & MATSUSHIMA, K. 1994a. Essential involvement of interleukin-8 (IL-8) in acute inflammation. *J Leukoc Biol,* 56**,** 559-64.

HARADA, K., TOYONAGA, A., MITSUYAMA, K., SASAKI, E. & TANIKAWA, K. 1994b. Role of cytokine-induced neutrophil chemoattractant, a member of the interleukin-8 family, in rat experimental colitis. *Digestion,* 55**,** 179-84.

HARDINGHAM, T. E. & FOSANG, A. J. 1992. Proteoglycans: many forms and many functions. *FASEB J,* 6**,** 861-70.

HARDY, M. M., SEIBERT, K., MANNING, P. T., CURRIE, M. G., WOERNER, B. M., EDWARDS, D., KOKI, A. & TRIPP, C. S. 2002. Cyclooxygenase 2-dependent prostaglandin E2 modulates cartilage proteoglycan degradation in human osteoarthritis explants. *Arthritis Rheum,* 46**,** 1789-803.

HARJACEK, M., DIAZ-CANO, S., DE MIGUEL, M., WOLFE, H., MALDONADO, C. A. & RABINOVICH, G. A. 2001. Expression of galectins-1 and -3 correlates with defective mononuclear cell apoptosis in patients with juvenile idiopathic arthritis. *J Rheumatol,* 28**,** 1914-22.

HARROLD, J. A., WIDDOWSON, P. S. & WILLIAMS, G. 2003. beta-MSH: a functional ligand that regulated energy homeostasis via hypothalamic MC4-R? *Peptides,* 24**,** 397-405.

HART, F. D. & BOARDMAN, P. L. 1963. Indomethacin: A New Non-Steroid Anti-Inflammatory Agent. *Br Med J,* 2**,** 965-70.

HARTMEYER, M., SCHOLZEN, T., BECHER, E., BHARDWAJ, R. S., SCHWARZ, T. & LUGER, T. A. 1997. Human dermal microvascular endothelial cells express the melanocortin receptor type 1 and produce increased levels of IL-8 upon stimulation with alpha-melanocyte-stimulating hormone. *J Immunol,* 159**,** 1930-7.

HAVERAN, L. A., SEHGAL, R., PORITZ, L. S., MCKENNA, K. J., STEWART, D. B. & KOLTUN, W. A. 2011. Infliximab and/or azathioprine in the treatment of Crohn's disease-like complications after IPAA. *Dis Colon Rectum,* 54**,** 15-20.

HEGEMANN, N., WONDIMU, A., KOHN, B., BRUNNBERG, L. & SCHMIDT, M. F. 2005. Cytokine profile in canine immune-mediated polyarthritis and osteoarthritis. *Vet Comp Orthop Traumatol,* 18**,** 67-72.

HEHLGANS, T. & PFEFFER, K. 2005. The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: players, rules and the games. *Immunology,* 115**,** 1-20.

HEILIGENHAUS, A., MISEROCCHI, E., HEINZ, C., GERLONI, V. & KOTANIEMI, K. 2011. Treatment of severe uveitis associated with juvenile idiopathic arthritis with anti-CD20 monoclonal antibody (rituximab). *Rheumatology (Oxford)*.

HEINRICH, P. C., BEHRMANN, I., HAAN, S., HERMANNS, H. M., MULLER-NEWEN, G. & SCHAPER, F. 2003. Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochem J,* 374**,** 1-20.

HEINRICH, P. C., BEHRMANN, I., MULLER-NEWEN, G., SCHAPER, F. & GRAEVE, L. 1998. Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *Biochem J,* 334 (Pt 2)**,** 297-314.

HENDERSON, N. C. & SETHI, T. 2009. The regulation of inflammation by galectin-3. *Immunol Rev,* 230**,** 160-71.

HENROTIN, Y. E., DE GROOTE, D. D., LABASSE, A. H., GASPAR, S. E., ZHENG, S. X., GEENEN, V. G. & REGINSTER, J. Y. 1996. Effects of exogenous IL-1 beta, TNF alpha, IL-6, IL-8 and LIF on cytokine production by human articular chondrocytes. *Osteoarthritis Cartilage,* 4**,** 163-73.

HERNBORG, J. & NILSSON, B. E. 1973. The relationship between osteophytes in the knee joint, osteoarthritis and aging. *Acta Orthop Scand,* 44**,** 69-74.

HEUCKENKAMP, P. U., MIKULIKOVA, D., TRNAVSKY, K., MITUSZOVA, M. & KEITEL, W. 1982. [Pathogenesis of gouty arthritis]. *Z Gesamte Inn Med,* 37**,** 424-8.

HILL, R. P., MACNEIL, S. & HAYCOCK, J. W. 2006. Melanocyte stimulating hormone peptides inhibit TNF-alpha signaling in human dermal fibroblast cells. *Peptides,* 27**,** 421-30.

HIRANO, T., ISHIHARA, K. & HIBI, M. 2000. Roles of STAT3 in mediating the cell growth, differentiation and survival signals relayed through the IL-6 family of cytokine receptors. *Oncogene,* 19**,** 2548-56.

HO, M. L., CHANG, J. K., WU, S. C., CHUNG, Y. H., CHEN, C. H., HUNG, S. H. & WANG, G. J. 2006. A novel terminal differentiation model of human articular chondrocytes in three-dimensional cultures mimicking chondrocytic changes in osteoarthritis. *Cell Biol Int,* 30**,** 288-94.

HOLLANDER, A. P., DICKINSON, S. C. & KAFIENAH, W. 2010. Stem cells and cartilage development: complexities of a simple tissue. *Stem Cells,* 28**,** 1992-6.

HRUBY, V. J., LU, D., SHARMA, S. D., CASTRUCCI, A. L., KESTERSON, R. A., AL-OBEIDI, F. A., HADLEY, M. E. & CONE, R. D. 1995. Cyclic lactam alpha-melanotropin analogues of Ac-Nle4-cyclo[Asp5, D-Phe7,Lys10] alpha-melanocyte-stimulating hormone-(4-10)-NH2 with bulky aromatic amino acids at position 7 show high antagonist potency and selectivity at specific melanocortin receptors. *J Med Chem,* 38**,** 3454-61.

HRYCAJ, P., STRATZ, T., KOVAC, C., MENNET, P. & MULLER, W. 1995. Microheterogeneity of acute phase proteins in patients with clinically active and clinically nonactive osteoarthritis. *Clin Rheumatol,* 14**,** 434-40.

HU, D. E., HORI, Y. & FAN, T. P. 1993. Interleukin-8 stimulates angiogenesis in rats. *Inflammation,* 17**,** 135-43.

HUI, W., ROWAN, A. D., RICHARDS, C. D. & CAWSTON, T. E. 2003. Oncostatin M in combination with tumor necrosis factor alpha induces cartilage damage and matrix metalloproteinase expression in vitro and in vivo. *Arthritis Rheum,* 48**,** 3404-18.

IANNONE, F., DE BARI, C., DELL'ACCIO, F., COVELLI, M., CANTATORE, F. P., PATELLA, V., LO BIANCO, G. & LAPADULA, G. 2001. Interleukin-10 and interleukin-10 receptor in human osteoarthritic and healthy chondrocytes. *Clin Exp Rheumatol,* 19**,** 139-45.

ICHIYAMA, T., SAKAI, T., CATANIA, A., BARSH, G. S., FURUKAWA, S. & LIPTON, J. M. 1999. Inhibition of peripheral NF-kappaB activation by central action of alpha-melanocyte-stimulating hormone. *J Neuroimmunol,* 99**,** 211-7.

IGNAR, D. M., ANDREWS, J. L., JANSEN, M., EILERT, M. M., PINK, H. M., LIN, P., SHERRILL, R. G., SZEWCZYK, J. R. & CONWAY, J. G. 2003. Regulation of TNF-alpha secretion by a specific melanocortin-1 receptor peptide agonist. *Peptides,* 24**,** 709-16.

ILIOPOULOS, D., MALIZOS, K. N., OIKONOMOU, P. & TSEZOU, A. 2008. Integrative microRNA and proteomic approaches identify novel osteoarthritis genes and their collaborative metabolic and inflammatory networks. *PLoS One,* 3**,** e3740.

IM, H. J., MUDDASANI, P., NATARAJAN, V., SCHMID, T. M., BLOCK, J. A., DAVIS, F., VAN WIJNEN, A. J. & LOESER, R. F. 2007. Basic fibroblast growth factor stimulates matrix metalloproteinase-13 via the molecular cross-talk between the mitogen-activated protein kinases and protein kinase Cdelta pathways in human adult articular chondrocytes. *J Biol Chem,* 282**,** 11110-21.

IMMENSCHUH, S., KIETZMANN, T., HINKE, V., WIEDERHOLD, M., KATZ, N. & MULLER-EBERHARD, U. 1998. The rat heme oxygenase-1 gene is transcriptionally induced via the protein kinase A signaling pathway in rat hepatocyte cultures. *Mol Pharmacol,* 53**,** 483-91.

IWAMOTO, G. K., MONICK, M. M., CLARK, B. D., AURON, P. E., STINSKI, M. F. & HUNNINGHAKE, G. W. 1990. Modulation of interleukin 1 beta gene expression by the immediate early genes of human cytomegalovirus. *J Clin Invest,* 85**,** 1853-7.

JACKSON, R. L., BUSCH, S. J. & CARDIN, A. D. 1991. Glycosaminoglycans: molecular properties, protein interactions, and role in physiological processes. *Physiol Rev,* 71**,** 481-539.

JASIN, H. E. 1983. Bacterial lipopolysaccharides induce in vitro degradation of cartilage matrix through chondrocyte activation. *J Clin Invest,* 72**,** 2014-9.

JENEY, V., BALLA, J., YACHIE, A., VARGA, Z., VERCELLOTTI, G. M., EATON, J. W. & BALLA, G. 2002. Pro-oxidant and cytotoxic effects of circulating heme. *Blood,* 100**,** 879-87.

KALDEN, D. H., SCHOLZEN, T., BRZOSKA, T. & LUGER, T. A. 1999. Mechanisms of the antiinflammatory effects of alpha-MSH. Role of transcription factor NF-kappa B and adhesion molecule expression. *Ann N Y Acad Sci,* 885**,** 254-61.

KAPOOR, M., MARTEL-PELLETIER, J., LAJEUNESSE, D., PELLETIER, J. P. & FAHMI, H. 2011. Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. *Nat Rev Rheumatol,* 7**,** 33-42.

KARAN, A., KARAN, M. A., VURAL, P., ERTEN, N., TASCIOGLU, C., AKSOY, C., CANBAZ, M. & ONCEL, A. 2003. Synovial fluid nitric oxide levels in patients with knee osteoarthritis. *Clin Rheumatol,* 22, 397-9.

KARGMAN, S., CHARLESON, S., CARTWRIGHT, M., FRANK, J., RIENDEAU, D., MANCINI, J., EVANS, J. & O'NEILL, G. 1996. Characterization of Prostaglandin G/H Synthase 1 and 2 in rat, dog, monkey, and human gastrointestinal tracts. *Gastroenterology,* 111**,** 445-54.

KARIN, M. 1995. The regulation of AP-1 activity by mitogen-activated protein kinases. *J Biol Chem,* 270**,** 16483-6.

KASK, A., MUTULIS, F., MUCENIECE, R., PAHKLA, R., MUTULE, I., WIKBERG, J. E., RAGO, L. & SCHIOTH, H. B. 1998. Discovery of a novel superpotent and selective melanocortin-4 receptor antagonist (HS024): evaluation in vitro and in vivo. *Endocrinology,* 139**,** 5006-14.

KASTNER, P., MARK, M. & CHAMBON, P. 1995. Nonsteroid nuclear receptors: what are genetic studies telling us about their role in real life? *Cell,* 83**,** 859-69.

KELLY, J. M., MOIR, A. J., CARLSON, K., YANG, Y., MACNEIL, S. & HAYCOCK, J. W. 2006. Immobilized alpha-melanocyte stimulating hormone 10-13 (GKPV) inhibits tumor necrosis factor-alpha stimulated NF-kappaB activity. *Peptides,* 27**,** 431-7.

KERR, J. F., WYLLIE, A. H. & CURRIE, A. R. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer,* 26**,** 239-57.

KERRIGAN, M. J. & HALL, A. C. 2005. Stimulation of regulatory volume decrease (RVD) by isolated bovine articular chondrocytes following F-actin disruption using latrunculin B. *Biorheology,* 42**,** 283-93.

KERRIGAN, M. J. & HALL, A. C. 2008. Control of chondrocyte regulatory volume decrease (RVD) by [Ca2+]i and cell shape. *Osteoarthritis Cartilage,* 16**,** 312-22.

KEVORKIAN, L., YOUNG, D. A., DARRAH, C., DONELL, S. T., SHEPSTONE, L., PORTER, S., BROCKBANK, S. M., EDWARDS, D. R., PARKER, A. E. & CLARK, I. M. 2004. Expression profiling of metalloproteinases and their inhibitors in cartilage. *Arthritis Rheum,* 50**,** 131-41.

KIM, H. A. & BLANCO, F. J. 2007. Cell death and apoptosis in osteoarthritic cartilage. *Curr Drug Targets,* 8**,** 333-45.

KIM, H. A., LEE, Y. J., SEONG, S. C., CHOE, K. W. & SONG, Y. W. 2000. Apoptotic chondrocyte death in human osteoarthritis. *J Rheumatol,* 27**,** 455-62.

KISHIMOTO, T., AKIRA, S., NARAZAKI, M. & TAGA, T. 1995. Interleukin-6 family of cytokines and gp130. *Blood,* 86**,** 1243-54.

KO, J. M., GOTTLIEB, A. B. & KERBLESKI, J. F. 2009. Induction and exacerbation of psoriasis with TNF-blockade therapy: a review and analysis of 127 cases. *J Dermatolog Treat,* 20**,** 100-8.

KOBAYASHI, M., SQUIRES, G. R., MOUSA, A., TANZER, M., ZUKOR, D. J., ANTONIOU, J., FEIGE, U. & POOLE, A. R. 2005. Role of interleukin-1 and tumor necrosis factor alpha in matrix degradation of human osteoarthritic cartilage. *Arthritis Rheum,* 52**,** 128-35.

KONDA, Y., GANTZ, I., DELVALLE, J., SHIMOTO, Y., MIWA, H. & YAMADA, T. 1994. Interaction of dual intracellular signaling pathways activated by the melanocortin-3 receptor. *J Biol Chem,* 269**,** 13162-6.

KONIG, H., PONTA, H., RAHMSDORF, H. J. & HERRLICH, P. 1992. Interference between pathway-specific transcription factors: glucocorticoids antagonize phorbol ester-induced AP-1 activity without altering AP-1 site occupation in vivo. *EMBO J,* 11**,** 2241-6.

KOOB, T. J. & VOGEL, K. G. 1987. Site-related variations in glycosaminoglycan content and swelling properties of bovine flexor tendon. *J Orthop Res,* 5**,** 414-24.

KORZUS, E., NAGASE, H., RYDELL, R. & TRAVIS, J. 1997. The mitogen-activated protein kinase and JAK-STAT signaling pathways are required for an oncostatin M-responsive element-mediated activation of matrix metalloproteinase 1 gene expression. *J Biol Chem,* 272**,** 1188-96.

KRANE, S. M. 1982. Collagenases and collagen degradation. *J Invest Dermatol,* 79 Suppl 1**,** 83s-86s.

KUNISCH, E., JANSEN, A., KOJIMA, F., LOFFLER, I., KAPOOR, M., KAWAI, S., RUBIO, I., CROFFORD, L. J. & KINNE, R. W. 2009. Prostaglandin E2 differentially modulates proinflammatory/prodestructive effects of TNF-alpha on synovial fibroblasts via specific E prostanoid receptors/cAMP. *J Immunol,* 183**,** 1328-36.

KURZ, B., LEMKE, A., KEHN, M., DOMM, C., PATWARI, P., FRANK, E. H., GRODZINSKY, A. J. & SCHUNKE, M. 2004. Influence of tissue maturation and antioxidants on the apoptotic response of articular cartilage after injurious compression. *Arthritis Rheum,* 50**,** 123-30.

LA, M., CAO, T. V., CERCHIARO, G., CHILTON, K., HIRABAYASHI, J., KASAI, K., OLIANI, S. M., CHERNAJOVSKY, Y. & PERRETTI, M. 2003. A novel biological activity for galectin-1: inhibition of leukocyte-endothelial cell interactions in experimental inflammation. *Am J Pathol,* 163**,** 1505-15.

LA, M., D'AMICO, M., BANDIERA, S., DI FILIPPO, C., OLIANI, S. M., GAVINS, F. N., FLOWER, R. J. & PERRETTI, M. 2001. Annexin 1 peptides protect against experimental myocardial ischemia-reperfusion: analysis of their mechanism of action. *FASEB J,* 15**,** 2247-56.

LAH, T. T., DURAN ALONSO, M. B. & VAN NOORDEN, C. J. 2006. Antiprotease therapy in cancer: hot or not? *Expert Opin Biol Ther,* 6**,** 257-79.

LAM, C. W., GETTING, S. J. & PERRETTI, M. 2005. In vitro and in vivo induction of heme oxygenase 1 in mouse macrophages following melanocortin receptor activation. *J Immunol,* 174**,** 2297-304.

LAM, C. W., PERRETTI, M. & GETTING, S. J. 2006. Melanocortin receptor signaling in RAW264.7 macrophage cell line. *Peptides,* 27**,** 404-12.

LAUTIER, D., LUSCHER, P. & TYRRELL, R. M. 1992. Endogenous glutathione levels modulate both constitutive and UVA radiation/hydrogen peroxide inducible expression of the human heme oxygenase gene. *Carcinogenesis,* 13**,** 227-32.

LAWRENCE, J. S., BREMNER, J. M. & BIER, F. 1966. Osteo-arthrosis. Prevalence in the population and relationship between symptoms and x-ray changes. *Ann Rheum Dis,* 25, 1-24.

LAWRENCE, J. S. & ZINN, W. M. 1970. Osteoarthrosis of the hip joint in Switzerland, in the United Kingdom, and in Jamaica, and its relationship to generalized osteoarthrosis. *Ann Rheum Dis,* 29, 191.

LAZZARONI, M. & BIANCHI PORRO, G. 2004. Gastrointestinal side-effects of traditional non-steroidal anti-inflammatory drugs and new formulations. *Aliment Pharmacol Ther,* 20 Suppl 2, 48-58.

LE, D. A., WU, Y., HUANG, Z., MATSUSHITA, K., PLESNILA, N., AUGUSTINACK, J. C., HYMAN, B. T., YUAN, J., KUIDA, K., FLAVELL, R. A. & MOSKOWITZ, M. A. 2002. Caspase activation and neuroprotection in caspase-3- deficient mice after in vivo cerebral ischemia and in vitro oxygen glucose deprivation. *Proc Natl Acad Sci U S A,* 99, 15188-93.

LEE, D. M. & WEINBLATT, M. E. 2001. Rheumatoid arthritis. *Lancet,* 358, 903-11.

LEE, H. H., SONG, I. H., FRIEDRICH, M., GAULIARD, A., DETERT, J., ROWERT, J., AUDRING, H., KARY, S., BURMESTER, G. R., STERRY, W. & WORM, M. 2007. Cutaneous side-effects in patients with rheumatic diseases during application of tumour necrosis factor-alpha antagonists. *Br J Dermatol,* 156, 486-91.

LEE, H. S., LEE, C. H., TSAI, H. C. & SALTER, D. M. 2009. Inhibition of cyclooxygenase 2 expression by diallyl sulfide on joint inflammation induced by urate crystal and IL-1beta. *Osteoarthritis Cartilage,* 17, 91-9.

LEE, T. S. & CHAU, L. Y. 2002. Heme oxygenase-1 mediates the anti-inflammatory effect of interleukin-10 in mice. *Nat Med,* 8, 240-6.

LEFKOWITZ, R. J., PITCHER, J., KRUEGER, K. & DAAKA, Y. 1998. Mechanisms of beta-adrenergic receptor desensitization and resensitization. *Adv Pharmacol,* 42, 416-20.

LEONI, G., PATEL, H. B., SAMPAIO, A. L., GAVINS, F. N., MURRAY, J. F., GRIECO, P., GETTING, S. J. & PERRETTI, M. 2008. Inflamed phenotype of the mesenteric microcirculation of melanocortin type 3 receptor-null mice after ischemia-reperfusion. *FASEB J,* 22, 4228-38.

LEPPA, S., SAFFRICH, R., ANSORGE, W. & BOHMANN, D. 1998. Differential regulation of c-Jun by ERK and JNK during PC12 cell differentiation. *EMBO J,* 17, 4404-13.

LERNER, A. B. & MCGUIRE, J. S. 1964. Melanocyte-Stimulating Hormone and Adrenocorticotrophic Hormone. Their Relation to Pigmentation. *N Engl J Med,* 270, 539-46.

LESPERANCE, L. M., GRAY, M. L. & BURSTEIN, D. 1992. Determination of fixed charge density in cartilage using nuclear magnetic resonance. *J Orthop Res,* 10, 1-13.

LEWIS, T. S., SHAPIRO, P. S. & AHN, N. G. 1998. Signal transduction through MAP kinase cascades. *Adv Cancer Res,* 74, 49-139.

LI, D., WU, Z., DUAN, Y., HAO, D., ZHANG, X., LUO, H., CHEN, B. & QIU, G. 2011. TNFalpha-mediated apoptosis in human osteoarthritic chondrocytes sensitized by PI3K-NF-kappaB inhibitor, not mTOR inhibitor. *Rheumatol Int*.

LI, G., WANG, S. & GELEHRTER, T. D. 2003. Identification of glucocorticoid receptor domains involved in transrepression of transforming growth factor-beta action. *J Biol Chem,* 278**,** 41779-88.

LI, T. F., ZUSCIK, M. J., IONESCU, A. M., ZHANG, X., ROSIER, R. N., SCHWARZ, E. M., DRISSI, H. & O'KEEFE, R. J. 2004. PGE2 inhibits chondrocyte differentiation through PKA and PKC signaling. *Exp Cell Res,* 300**,** 159-69.

LIM, H. & KIM, H. P. 2011. Matrix metalloproteinase-13 expression in IL-1beta-treated chondrocytes by activation of the p38 MAPK/c-Fos/AP-1 and JAK/STAT pathways. *Arch Pharm Res,* 34**,** 109-17.

LIN, A., MINDEN, A., MARTINETTO, H., CLARET, F. X., LANGE-CARTER, C., MERCURIO, F., JOHNSON, G. L. & KARIN, M. 1995. Identification of a dual specificity kinase that activates the Jun kinases and p38-Mpk2. *Science,* 268**,** 286-90.

LIPTON, J. M. & CATANIA, A. 1997. Anti-inflammatory actions of the neuroimmunomodulator alpha-MSH. *Immunol Today,* 18**,** 140-5.

LIPTON, J. M., ZHAO, H., ICHIYAMA, T., BARSH, G. S. & CATANIA, A. 1999. Mechanisms of antiinflammatory action of alpha-MSH peptides. In vivo and in vitro evidence. *Ann N Y Acad Sci,* 885**,** 173-82.

LIU-BRYAN, R. & TERKELTAUB, R. 2010. Chondrocyte innate immune myeloid differentiation factor 88-dependent signaling drives procatabolic effects of the endogenous Toll-like receptor 2/Toll-like receptor 4 ligands low molecular weight hyaluronan and high mobility group box chromosomal protein 1 in mice. *Arthritis Rheum,* 62**,** 2004-12.

LIU, Y. H., D'AMBROSIO, M., LIAO, T. D., PENG, H., RHALEB, N. E., SHARMA, U., ANDRE, S., GABIUS, H. J. & CARRETERO, O. A. 2009. N-acetyl-seryl-aspartyl-lysyl-proline prevents cardiac remodeling and dysfunction induced by galectin-3, a mammalian adhesion/growth-regulatory lectin. *Am J Physiol Heart Circ Physiol,* 296**,** H404-12.

LOCKSLEY, R. M., KILLEEN, N. & LENARDO, M. J. 2001. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell,* 104**,** 487-501.

LOPEZ-ARMADA, M. J., CARAMES, B., LIRES-DEAN, M., CILLERO-PASTOR, B., RUIZ-ROMERO, C., GALDO, F. & BLANCO, F. J. 2006. Cytokines, tumor necrosis factor-alpha and interleukin-1beta, differentially regulate apoptosis in osteoarthritis cultured human chondrocytes. *Osteoarthritis Cartilage,* 14**,** 660-9.

LOTZ, M., TERKELTAUB, R. & VILLIGER, P. M. 1992. Cartilage and joint inflammation. Regulation of IL-8 expression by human articular chondrocytes. *J Immunol,* 148**,** 466-73.

LU, D., WILLARD, D., PATEL, I. R., KADWELL, S., OVERTON, L., KOST, T., LUTHER, M., CHEN, W., WOYCHIK, R. P., WILKISON, W. O. & ET AL. 1994. Agouti protein is an antagonist of the melanocyte-stimulating-hormone receptor. *Nature,* 371**,** 799-802.

LUGER, T. A., BRZOSKA, T., SCHOLZEN, T. E., KALDEN, D. H., SUNDERKOTTER, C., ARMSTRONG, C. & ANSEL, J. 2000. The role of alpha-MSH as a modulator of cutaneous inflammation. *Ann N Y Acad Sci,* 917**,** 232-8.

LUSTER, A. D. 1998. Chemokines--chemotactic cytokines that mediate inflammation. *N Engl J Med,* 338**,** 436-45.

MACALUSO, A., MCCOY, D., CERIANI, G., WATANABE, T., BILTZ, J., CATANIA, A. & LIPTON, J. M. 1994. Antiinflammatory influences of alpha-MSH molecules: central neurogenic and peripheral actions. *J Neurosci,* 14**,** 2377-82.

MACCOUX, L. J., SALWAY, F., DAY, P. J. & CLEMENTS, D. N. 2007. Expression profiling of select cytokines in canine osteoarthritis tissues. *Vet Immunol Immunopathol,* 118**,** 59-67.

MANDRIKA, I., MUCENIECE, R. & WIKBERG, J. E. 2001. Effects of melanocortin peptides on lipopolysaccharide/interferon-gamma-induced NF-kappaB DNA binding and nitric oxide production in macrophage-like RAW 264.7 cells: evidence for dual mechanisms of action. *Biochem Pharmacol,* 61**,** 613-21.

MANGELSDORF, D. J., THUMMEL, C., BEATO, M., HERRLICH, P., SCHUTZ, G., UMESONO, K., BLUMBERG, B., KASTNER, P., MARK, M., CHAMBON, P. & EVANS, R. M. 1995. The nuclear receptor superfamily: the second decade. *Cell,* 83**,** 835-9.

MANKIN, H. J. & LIPPIELLO, L. 1970. Biochemical and metabolic abnormalities in articular cartilage from osteo-arthritic human hips. *J Bone Joint Surg Am,* 52**,** 424-34.

MARCH, C. J., MOSLEY, B., LARSEN, A., CERRETTI, D. P., BRAEDT, G., PRICE, V., GILLIS, S., HENNEY, C. S., KRONHEIM, S. R., GRABSTEIN, K. & ET AL. 1985. Cloning, sequence and expression of two distinct human interleukin-1 complementary DNAs. *Nature,* 315**,** 641-7.

MARKS, R. & ALLEGRANTE, J. P. 2002a. Body mass indices in patients with disabling hip osteoarthritis. *Arthritis Res,* 4**,** 112-6.

MARKS, R. & ALLEGRANTE, J. P. 2002b. Comorbid disease profiles of adults with end-stage hip osteoarthritis. *Med Sci Monit,* 8**,** CR305-9.

MAROUDAS, A. I. 1976. Balance between swelling pressure and collagen tension in normal and degenerate cartilage. *Nature,* 260**,** 808-9.

MARTEL-PELLETIER, J., ALAAEDDINE, N. & PELLETIER, J. P. 1999. Cytokines and their role in the pathophysiology of osteoarthritis. *Front Biosci,* 4**,** D694-703.

MARTEL-PELLETIER, J. & PELLETIER, J. P. 2010. Is osteoarthritis a disease involving only cartilage or other articular tissues? *Eklem Hastalik Cerrahisi,* 21**,** 2-14.

MARTEL-PELLETIER, J., WELSCH, D. J. & PELLETIER, J. P. 2001. Metalloproteases and inhibitors in arthritic diseases. *Best Pract Res Clin Rheumatol,* 15**,** 805-29.

MARTIGNONI, E., GODI, L., PACCHETTI, C., BERARDESCA, E., VIGNOLI, G. P., ALBANI, G., MANCINI, F. & NAPPI, G. 1997. Is seborrhea a sign of autonomic impairment in Parkinson's disease? *J Neural Transm,* 104**,** 1295-304.

MARTIN, L. W., CATANIA, A., HILTZ, M. E. & LIPTON, J. M. 1991. Neuropeptide alpha-MSH antagonizes IL-6- and TNF-induced fever. *Peptides,* 12**,** 297-9.

MATSUNO, H., YUDOH, K., WATANABE, Y., NAKAZAWA, F., AONO, H. & KIMURA, T. 2001. Stromelysin-1 (MMP-3) in synovial fluid of patients with rheumatoid arthritis has potential to cleave membrane bound Fas ligand. *J Rheumatol,* 28**,** 22-8.

MATTHEWS, L. S., SONSTEGARD, D. A. & HENKE, J. A. 1977. Load bearing characteristics of the patello-femoral joint. *Acta Orthop Scand,* 48**,** 511-6.

MCADAM, B. F., CATELLA-LAWSON, F., MARDINI, I. A., KAPOOR, S., LAWSON, J. A. & FITZGERALD, G. A. 1999. Systemic biosynthesis of prostacyclin by cyclooxygenase (COX)-2: the human pharmacology of a selective inhibitor of COX-2. *Proc Natl Acad Sci U S A,* 96**,** 272-7.

MCCARTHY, C., CUSHNAGHAN, J. & DIEPPE, P. 1994. The predictive role of scintigraphy in radiographic osteoarthritis of the hand. *Osteoarthritis Cartilage,* 2**,** 25-8.

MCCARTY, D. J., JR., PHELPS, P. & PYENSON, J. 1966. Crystal-induced inflammation in canine joints. I. An experimental model with quantification of the host response. *J Exp Med,* 124**,** 99-114.

MCGILL, N. W. & DIEPPE, P. A. 1991. Evidence for a promoter of urate crystal formation in gouty synovial fluid. *Ann Rheum Dis,* 50**,** 558-61.

MCWILLIAMS, D. F., LEEB, B. F., MUTHURI, S. G., DOHERTY, M. & ZHANG, W. 2011. Occupational risk factors for osteoarthritis of the knee: a meta-analysis. *Osteoarthritis Cartilage.*

MELCHIORRI, C., MELICONI, R., FRIZZIERO, L., SILVESTRI, T., PULSATELLI, L., MAZZETTI, I., BORZI, R. M., UGUCCIONI, M. & FACCHINI, A. 1998. Enhanced and coordinated in vivo expression of inflammatory cytokines and nitric oxide synthase by chondrocytes from patients with osteoarthritis. *Arthritis Rheum,* 41**,** 2165-74.

MENDES, A. F., CARAMONA, M. M., CARVALHO, A. P. & LOPES, M. C. 2003a. Differential roles of hydrogen peroxide and superoxide in mediating IL-1-induced NF-kappa B activation and iNOS expression in bovine articular chondrocytes. *J Cell Biochem,* 88**,** 783-93.

MENDES, A. F., CARAMONA, M. M., CARVALHO, A. P. & LOPES, M. C. 2003b. Hydrogen peroxide mediates interleukin-1beta-induced AP-1 activation in

articular chondrocytes: implications for the regulation of iNOS expression. *Cell Biol Toxicol,* 19**,** 203-14.

MENGSHOL, J. A., VINCENTI, M. P., COON, C. I., BARCHOWSKY, A. & BRINCKERHOFF, C. E. 2000. Interleukin-1 induction of collagenase 3 (matrix metalloproteinase 13) gene expression in chondrocytes requires p38, c-Jun N-terminal kinase, and nuclear factor kappaB: differential regulation of collagenase 1 and collagenase 3. *Arthritis Rheum,* 43**,** 801-11.

MEYERS, J. A., SANCHEZ, D., ELWELL, L. P. & FALKOW, S. 1976. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. *J Bacteriol,* 127**,** 1529-37.

MIAN, B. M., DINNEY, C. P., BERMEJO, C. E., SWEENEY, P., TELLEZ, C., YANG, X. D., GUDAS, J. M., MCCONKEY, D. J. & BAR-ELI, M. 2003. Fully human anti-interleukin 8 antibody inhibits tumor growth in orthotopic bladder cancer xenografts via down-regulation of matrix metalloproteases and nuclear factor-kappaB. *Clin Cancer Res,* 9**,** 3167-75.

MIDDLETON, J., MANTHEY, A. & TYLER, J. 1996. Insulin-like growth factor (IGF) receptor, IGF-I, interleukin-1 beta (IL-1 beta), and IL-6 mRNA expression in osteoarthritic and normal human cartilage. *J Histochem Cytochem,* 44**,** 133-41.

MITCHELL, P. G., MAGNA, H. A., REEVES, L. M., LOPRESTI-MORROW, L. L., YOCUM, S. A., ROSNER, P. J., GEOGHEGAN, K. F. & HAMBOR, J. E. 1996. Cloning, expression, and type II collagenolytic activity of matrix metalloproteinase-13 from human osteoarthritic cartilage. *J Clin Invest,* 97**,** 761-8.

MIYAMOTO, M., ITO, H., MUKAI, S., KOBAYASHI, T., YAMAMOTO, H., KOBAYASHI, M., MARUYAMA, T., AKIYAMA, H. & NAKAMURA, T. 2003. Simultaneous stimulation of EP2 and EP4 is essential to the effect of prostaglandin E2 in chondrocyte differentiation. *Osteoarthritis Cartilage,* 11**,** 644-52.

MIZEL, S. B., DAYER, J. M., KRANE, S. M. & MERGENHAGEN, S. E. 1981. Stimulation of rheumatoid synovial cell collagenase and prostaglandin production by partially purified lymphocyte-activating factor (interleukin 1). *Proc Natl Acad Sci U S A,* 78**,** 2474-7.

MODI, W. S., DEAN, M., SEUANEZ, H. N., MUKAIDA, N., MATSUSHIMA, K. & O'BRIEN, S. J. 1990. Monocyte-derived neutrophil chemotactic factor (MDNCF/IL-8) resides in a gene cluster along with several other members of the platelet factor 4 gene superfamily. *Hum Genet,* 84**,** 185-7.

MOLDOVAN, F., PELLETIER, J. P., JOLICOEUR, F. C., CLOUTIER, J. M. & MARTEL-PELLETIER, J. 2000. Diacerhein and rhein reduce the ICE-induced IL-1beta and IL-18 activation in human osteoarthritic cartilage. *Osteoarthritis Cartilage,* 8**,** 186-96.

MORALES, T. I., WAHL, L. M. & HASCALL, V. C. 1984. The effect of bacterial lipopolysaccharides on the biosynthesis and release of proteoglycans from calf articular cartilage cultures. *J Biol Chem,* 259**,** 6720-9.

MOSMANN, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods,* 65**,** 55-63.

MOUNTJOY, K. G., MORTRUD, M. T., LOW, M. J., SIMERLY, R. B. & CONE, R. D. 1994. Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the brain. *Mol Endocrinol,* 8**,** 1298-308.

MOUNTJOY, K. G., ROBBINS, L. S., MORTRUD, M. T. & CONE, R. D. 1992. The cloning of a family of genes that encode the melanocortin receptors. *Science,* 257**,** 1248-51.

MUDDASANI, P., NORMAN, J. C., ELLMAN, M., VAN WIJNEN, A. J. & IM, H. J. 2007. Basic fibroblast growth factor activates the MAPK and NFkappaB pathways that converge on Elk-1 to control production of matrix metalloproteinase-13 by human adult articular chondrocytes. *J Biol Chem,* 282**,** 31409-21.

MUIR, H., BULLOUGH, P. & MAROUDAS, A. 1970. The distribution of collagen in human articular cartilage with some of its physiological implications. *J Bone Joint Surg Br,* 52**,** 554-63.

MUKAIDA, N., MORITA, M., ISHIKAWA, Y., RICE, N., OKAMOTO, S., KASAHARA, T. & MATSUSHIMA, K. 1994. Novel mechanism of glucocorticoid-mediated gene repression. Nuclear factor-kappa B is target for glucocorticoid-mediated interleukin 8 gene repression. *J Biol Chem,* 269**,** 13289-95.

MUKAIDA, N., ZACHARIAE, C. C., GUSELLA, G. L. & MATSUSHIMA, K. 1991. Dexamethasone inhibits the induction of monocyte chemotactic-activating factor production by IL-1 or tumor necrosis factor. *J Immunol,* 146**,** 1212-5.

MUKHERJEE, D., NISSEN, S. E. & TOPOL, E. J. 2001. Risk of cardiovascular events associated with selective COX-2 inhibitors. *JAMA,* 286**,** 954-9.

MURAKAMI, M., NAKATANI, Y., KUWATA, H. & KUDO, I. 2000. Cellular components that functionally interact with signaling phospholipase A(2)s. *Biochim Biophys Acta,* 1488**,** 159-66.

MUSUMECI, G., LORETO, C., CARNAZZA, M. L. & MARTINEZ, G. 2011. Characterization of apoptosis in articular cartilage derived from the knee joints of patients with osteoarthritis. *Knee Surg Sports Traumatol Arthrosc,* 19**,** 307-13.

NAKANISHI, S., INOUE, A., KITA, T., NAKAMURA, M., CHANG, A. C., COHEN, S. N. & NUMA, S. 1979. Nucleotide sequence of cloned cDNA for bovine corticotropin-beta-lipotropin precursor. *Nature,* 278**,** 423-7.

NEUMANN ANDERSEN, G., NAGAEVA, O., MANDRIKA, I., PETROVSKA, R., MUCENIECE, R., MINCHEVA-NILSSON, L. & WIKBERG, J. E. 2001. MC(1) receptors are constitutively expressed on leucocyte subpopulations

with antigen presenting and cytotoxic functions. *Clin Exp Immunol,* 126**,** 441-6.

NICHOLSON, D. W. & THORNBERRY, N. A. 1997. Caspases: killer proteases. *Trends Biochem Sci,* 22**,** 299-306.

NORLING, L. V., PERRETTI, M. & COOPER, D. 2009. Endogenous galectins and the control of the host inflammatory response. *J Endocrinol,* 201**,** 169-84.

NORRBY, K. 1996. Interleukin-8 and de novo mammalian angiogenesis. *Cell Prolif,* 29**,** 315-23.

NOTOYA, K., JOVANOVIC, D. V., REBOUL, P., MARTEL-PELLETIER, J., MINEAU, F. & PELLETIER, J. P. 2000. The induction of cell death in human osteoarthritis chondrocytes by nitric oxide is related to the production of prostaglandin E2 via the induction of cyclooxygenase-2. *J Immunol,* 165**,** 3402-10.

OHSHIMA, S., KUCHEN, S., SEEMAYER, C. A., KYBURZ, D., HIRT, A., KLINZING, S., MICHEL, B. A., GAY, R. E., LIU, F. T., GAY, S. & NEIDHART, M. 2003. Galectin 3 and its binding protein in rheumatoid arthritis. *Arthritis Rheum,* 48**,** 2788-95.

OLLMANN, M. M., WILSON, B. D., YANG, Y. K., KERNS, J. A., CHEN, Y., GANTZ, I. & BARSH, G. S. 1997. Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. *Science,* 278**,** 135-8.

OPPENHEIM, J. J., MATSUSHIMA, K., YOSHIMURA, T., LEONARD, E. J. & NETA, R. 1989. Relationship between interleukin 1 (IL1), tumor necrosis factor (TNF) and a neutrophil attracting peptide (NAP-1). *Agents Actions,* 26**,** 134-40.

PALLADINO, M. A., BAHJAT, F. R., THEODORAKIS, E. A. & MOLDAWER, L. L. 2003. Anti-TNF-alpha therapies: the next generation. *Nat Rev Drug Discov,* 2**,** 736-46.

PALMER, G., GUERNE, P. A., MEZIN, F., MARET, M., GUICHEUX, J., GOLDRING, M. B. & GABAY, C. 2002. Production of interleukin-1 receptor antagonist by human articular chondrocytes. *Arthritis Res,* 4**,** 226-31.

PARADA-TURSKA, J. & MAJDAN, M. 2005. [The musculoskeletal system in diabetic patients]. *Postepy Hig Med Dosw (Online),* 59**,** 236-44.

PATEL, H. B., BOMBARDIERI, M., SAMPAIO, A. L., D'ACQUISTO, F., GRAY, M., GRIECO, P., GETTING, S. J., PITZALIS, C. & PERRETTI, M. 2010. Anti-inflammatory and antiosteoclastogenesis properties of endogenous melanocortin receptor type 3 in experimental arthritis. *FASEB J,* 24**,** 4835-43.

PELAIA, G., VATRELLA, A., CUDA, G., MASELLI, R. & MARSICO, S. A. 2003. Molecular mechanisms of corticosteroid actions in chronic inflammatory airway diseases. *Life Sci,* 72**,** 1549-61.

PELLETIER, J. P., FERNANDES, J. C., JOVANOVIC, D. V., REBOUL, P. & MARTEL-PELLETIER, J. 2001a. Chondrocyte death in experimental osteoarthritis is mediated by MEK 1/2 and p38 pathways: role of cyclooxygenase-2 and inducible nitric oxide synthase. *J Rheumatol,* 28**,** 2509-19.

PELLETIER, J. P., MARTEL-PELLETIER, J. & ABRAMSON, S. B. 2001b. Osteoarthritis, an inflammatory disease: potential implication for the selection of new therapeutic targets. *Arthritis Rheum,* 44**,** 1237-47.

PELLETIER, J. P., ROUGHLEY, P. J., DIBATTISTA, J. A., MCCOLLUM, R. & MARTEL-PELLETIER, J. 1991. Are cytokines involved in osteoarthritic pathophysiology? *Semin Arthritis Rheum,* 20**,** 12-25.

PERKINS, J. P. 1991. Mechanisms of ligand-induced desensitization of beta adrenergic receptors. *Perkins JP, ed. The beta-adrenergic receptor.* Clifton NJ: Humana Press.

PERONE, M. J., BERTERA, S., TAWADROUS, Z. S., SHUFESKY, W. J., PIGANELLI, J. D., BAUM, L. G., TRUCCO, M. & MORELLI, A. E. 2006. Dendritic cells expressing transgenic galectin-1 delay onset of autoimmune diabetes in mice. *J Immunol,* 177**,** 5278-89.

PERRETTI, M., AHLUWALIA, A., HARRIS, J. G., GOULDING, N. J. & FLOWER, R. J. 1993. Lipocortin-1 fragments inhibit neutrophil accumulation and neutrophil-dependent edema in the mouse. A qualitative comparison with an anti-CD11b monoclonal antibody. *J Immunol,* 151**,** 4306-14.

PERRETTI, M. & GAVINS, F. N. 2003. Annexin 1: an endogenous anti-inflammatory protein. *News Physiol Sci,* 18**,** 60-4.

PERRETTI, M., INGEGNOLI, F., WHELLER, S. K., BLADES, M. C., SOLITO, E. & PITZALIS, C. 2002. Annexin 1 modulates monocyte-endothelial cell interaction in vitro and cell migration in vivo in the human SCID mouse transplantation model. *J Immunol,* 169**,** 2085-92.

PHILIP, J. G., FLOWER, R. J. & BUCKINGHAM, J. C. 1997. Glucocorticoids modulate the cellular disposition of lipocortin 1 in the rat brain in vivo and in vitro. *Neuroreport,* 8**,** 1871-6.

PICKVANCE, E. A., OEGEMA, T. R., JR. & THOMPSON, R. C., JR. 1993. Immunolocalization of selected cytokines and proteases in canine articular cartilage after transarticular loading. *J Orthop Res,* 11**,** 313-23.

POOLE, C. A., AYAD, S. & GILBERT, R. T. 1992. Chondrons from articular cartilage. V. Immunohistochemical evaluation of type VI collagen organisation in isolated chondrons by light, confocal and electron microscopy. *J Cell Sci,* 103 (Pt 4)**,** 1101-10.

QUINN, T. M., GRODZINSKY, A. J., HUNZIKER, E. B. & SANDY, J. D. 1998. Effects of injurious compression on matrix turnover around individual cells in calf articular cartilage explants. *J Orthop Res,* 16**,** 490-9.

QUINN, T. M., MAUNG, A. A., GRODZINSKY, A. J., HUNZIKER, E. B. & SANDY, J. D. 1999. Physical and biological regulation of proteoglycan

turnover around chondrocytes in cartilage explants. Implications for tissue degradation and repair. *Ann N Y Acad Sci,* 878**,** 420-41.

RAAP, U., BRZOSKA, T., SOHL, S., PATH, G., EMMEL, J., HERZ, U., BRAUN, A., LUGER, T. & RENZ, H. 2003. Alpha-melanocyte-stimulating hormone inhibits allergic airway inflammation. *J Immunol,* 171**,** 353-9.

RABINOVICH, G. A., DALY, G., DREJA, H., TAILOR, H., RIERA, C. M., HIRABAYASHI, J. & CHERNAJOVSKY, Y. 1999. Recombinant galectin-1 and its genetic delivery suppress collagen-induced arthritis via T cell apoptosis. *J Exp Med,* 190**,** 385-98.

RAI, M. F., RACHAKONDA, P. S., MANNING, K., VORWERK, B., BRUNNBERG, L., KOHN, B. & SCHMIDT, M. F. 2008. Quantification of cytokines and inflammatory mediators in a three-dimensional model of inflammatory arthritis. *Cytokine,* 42**,** 8-17.

RAJORA, N., BOCCOLI, G., BURNS, D., SHARMA, S., CATANIA, A. P. & LIPTON, J. M. 1997a. alpha-MSH modulates local and circulating tumor necrosis factor-alpha in experimental brain inflammation. *J Neurosci,* 17**,** 2181-6.

RAJORA, N., BOCCOLI, G., CATANIA, A. & LIPTON, J. M. 1997b. alpha-MSH modulates experimental inflammatory bowel disease. *Peptides,* 18**,** 381-5.

RAJORA, N., CERIANI, G., CATANIA, A., STAR, R. A., MURPHY, M. T. & LIPTON, J. M. 1996. alpha-MSH production, receptors, and influence on neopterin in a human monocyte/macrophage cell line. *J Leukoc Biol,* 59**,** 248-53.

RANNOU, F., FRANCOIS, M., CORVOL, M. T. & BERENBAUM, F. 2006. Cartilage breakdown in rheumatoid arthritis. *Joint Bone Spine,* 73**,** 29-36.

RAO, P. & KNAUS, E. E. 2008. Evolution of nonsteroidal anti-inflammatory drugs (NSAIDs): cyclooxygenase (COX) inhibition and beyond. *J Pharm Pharm Sci,* 11**,** 81s-110s.

RAY, A. & PREFONTAINE, K. E. 1994. Physical association and functional antagonism between the p65 subunit of transcription factor NF-kappa B and the glucocorticoid receptor. *Proc Natl Acad Sci U S A,* 91**,** 752-6.

REBOUL, P., PELLETIER, J. P., TARDIF, G., CLOUTIER, J. M. & MARTEL-PELLETIER, J. 1996. The new collagenase, collagenase-3, is expressed and synthesized by human chondrocytes but not by synoviocytes. A role in osteoarthritis. *J Clin Invest,* 97**,** 2011-9.

RECKLIES, A. D. & GOLDS, E. E. 1992. Induction of synthesis and release of interleukin-8 from human articular chondrocytes and cartilage explants. *Arthritis Rheum,* 35**,** 1510-9.

REDONDO, P., GARCIA-FONCILLAS, J., OKROUJNOV, I. & BANDRES, E. 1998. Alpha-MSH regulates interleukin-10 expression by human keratinocytes. *Arch Dermatol Res,* 290**,** 425-8.

REGINATO, A. M., SANZ-RODRIGUEZ, C., DIAZ, A., DHARMAVARAM, R. M. & JIMENEZ, S. A. 1993. Transcriptional modulation of cartilage-specific

collagen gene expression by interferon gamma and tumour necrosis factor alpha in cultured human chondrocytes. *Biochem J,* 294 (Pt 3)**,** 761-9.

REID, C. R., BUSH, P. M., CUMMINGS, N. H., MCMULLIN, D. L. & DURRANI, S. K. 2010. A review of occupational knee disorders. *J Occup Rehabil,* 20**,** 489-501.

RICHARDSON, D. W. & DODGE, G. R. 2003. Dose-dependent effects of corticosteroids on the expression of matrix-related genes in normal and cytokine-treated articular chondrocytes. *Inflamm Res,* 52**,** 39-49.

RILEY, J. K., TAKEDA, K., AKIRA, S. & SCHREIBER, R. D. 1999. Interleukin-10 receptor signaling through the JAK-STAT pathway. Requirement for two distinct receptor-derived signals for anti-inflammatory action. *J Biol Chem,* 274**,** 16513-21.

RITTER, J., KERR, L. D., VALERIANO-MARCET, J. & SPIERA, H. 1994. ACTH revisited: effective treatment for acute crystal induced synovitis in patients with multiple medical problems. *J Rheumatol,* 21**,** 696-9.

ROACH, H. I., AIGNER, T. & KOURI, J. B. 2004. Chondroptosis: a variant of apoptotic cell death in chondrocytes? *Apoptosis,* 9**,** 265-77.

ROBERTSON, C. M., PENNOCK, A. T., HARWOOD, F. L., POMERLEAU, A. C., ALLEN, R. T. & AMIEL, D. 2006. Characterization of pro-apoptotic and matrix-degradative gene expression following induction of osteoarthritis in mature and aged rabbits. *Osteoarthritis Cartilage,* 14**,** 471-6.

ROSELLI-REHFUSS, L., MOUNTJOY, K. G., ROBBINS, L. S., MORTRUD, M. T., LOW, M. J., TATRO, J. B., ENTWISTLE, M. L., SIMERLY, R. B. & CONE, R. D. 1993. Identification of a receptor for gamma melanotropin and other proopiomelanocortin peptides in the hypothalamus and limbic system. *Proc Natl Acad Sci U S A,* 90**,** 8856-60.

ROSSIGNOL, M., LECLERC, A., ALLAERT, F. A., ROZENBERG, S., VALAT, J. P., AVOUAC, B., COSTE, P., LITVAK, E. & HILLIQUIN, P. 2005. Primary osteoarthritis of hip, knee, and hand in relation to occupational exposure. *Occup Environ Med,* 62**,** 772-7.

ROVIEZZO, F., GETTING, S. J., PAUL-CLARK, M. J., YONA, S., GAVINS, F. N., PERRETTI, M., HANNON, R., CROXTALL, J. D., BUCKINGHAM, J. C. & FLOWER, R. J. 2002. The annexin-1 knockout mouse: what it tells us about the inflammatory response. *J Physiol Pharmacol,* 53**,** 541-53.

RUTTER, G. A., WHITE, M. R. & TAVARE, J. M. 1995. Involvement of MAP kinase in insulin signalling revealed by non-invasive imaging of luciferase gene expression in single living cells. *Curr Biol,* 5**,** 890-9.

SAAMANEN, A. M., TAMMI, M., KIVIRANTA, I., JURVELIN, J. & HELMINEN, H. J. 1987. Maturation of proteoglycan matrix in articular cartilage under increased and decreased joint loading. A study in young rabbits. *Connect Tissue Res,* 16**,** 163-75.

SAILOR, L. Z., HEWICK, R. M. & MORRIS, E. A. 1996. Recombinant human bone morphogenetic protein-2 maintains the articular chondrocyte phenotype in long-term culture. *J Orthop Res,* 14**,** 937-45.

SAMPIERI, C. L., NUTTALL, R. K., YOUNG, D. A., GOLDSPINK, D., CLARK, I. M. & EDWARDS, D. R. 2008. Activation of p38 and JNK MAPK pathways abrogates requirement for new protein synthesis for phorbol ester mediated induction of select MMP and TIMP genes. *Matrix Biol,* 27**,** 128-38.

SANTUCCI, L., FIORUCCI, S., RUBINSTEIN, N., MENCARELLI, A., PALAZZETTI, B., FEDERICI, B., RABINOVICH, G. A. & MORELLI, A. 2003. Galectin-1 suppresses experimental colitis in mice. *Gastroenterology,* 124**,** 1381-94.

SCHACKE, H., SCHOTTELIUS, A., DOCKE, W. D., STREHLKE, P., JAROCH, S., SCHMEES, N., REHWINKEL, H., HENNEKES, H. & ASADULLAH, K. 2004. Dissociation of transactivation from transrepression by a selective glucocorticoid receptor agonist leads to separation of therapeutic effects from side effects. *Proc Natl Acad Sci U S A,* 101**,** 227-32.

SCHEINMAN, R. I., COGSWELL, P. C., LOFQUIST, A. K. & BALDWIN, A. S., JR. 1995. Role of transcriptional activation of I kappa B alpha in mediation of immunosuppression by glucocorticoids. *Science,* 270**,** 283-6.

SCHIOTH, H. B., MUCENIECE, R., MUTULIS, F., BOUIFROURI, A. A., MUTULE, I. & WIKBERG, J. E. 1999. Further pharmacological characterization of the selective melanocortin 4 receptor antagonist HS014: comparison with SHU9119. *Neuropeptides,* 33**,** 191-6.

SCHIOTH, H. B., MUCENIECE, R. & WIKBERG, J. E. 1996. Characterisation of the melanocortin 4 receptor by radioligand binding. *Pharmacol Toxicol,* 79**,** 161-5.

SCHMIDT, N., PAUTZ, A., ART, J., RAUSCHKOLB, P., JUNG, M., ERKEL, G., GOLDRING, M. B. & KLEINERT, H. 2010. Transcriptional and post-transcriptional regulation of iNOS expression in human chondrocytes. *Biochem Pharmacol,* 79**,** 722-32.

SCHOLZEN, T. E., SUNDERKOTTER, C., KALDEN, D. H., BRZOSKA, T., FASTRICH, M., FISBECK, T., ARMSTRONG, C. A., ANSEL, J. C. & LUGER, T. A. 2003. Alpha-melanocyte stimulating hormone prevents lipopolysaccharide-induced vasculitis by down-regulating endothelial cell adhesion molecule expression. *Endocrinology,* 144**,** 360-70.

SCHONEVELD, O. J., GAEMERS, I. C. & LAMERS, W. H. 2004. Mechanisms of glucocorticoid signalling. *Biochim Biophys Acta,* 1680**,** 114-28.

SCHUERWEGH, A. J., DOMBRECHT, E. J., STEVENS, W. J., VAN OFFEL, J. F., BRIDTS, C. H. & DE CLERCK, L. S. 2003. Influence of pro-inflammatory (IL-1 alpha, IL-6, TNF-alpha, IFN-gamma) and anti-inflammatory (IL-4) cytokines on chondrocyte function. *Osteoarthritis Cartilage,* 11**,** 681-7.

SEKI, M., SAKATA, K. M., OOMIZU, S., ARIKAWA, T., SAKATA, A., UENO, M., NOBUMOTO, A., NIKI, T., SAITA, N., ITO, K., DAI, S. Y., KATOH, S., NISHI, N., TSUKANO, M., ISHIKAWA, K., YAMAUCHI, A., KUCHROO, V. & HIRASHIMA, M. 2007. Beneficial effect of galectin 9 on rheumatoid

arthritis by induction of apoptosis of synovial fibroblasts. *Arthritis Rheum,* 56**,** 3968-76.

SEVCIC, K., ORBAN, I., BRODSZKY, V., BAZSO, A., BALOGH, Z., POOR, G. & KISS, E. 2011. Experiences with tumour necrosis factor-{alpha} inhibitors in patients with juvenile idiopathic arthritis: Hungarian data from the National Institute of Rheumatology and Physiotherapy Registry. *Rheumatology (Oxford).*

SHARIF, M., WHITEHOUSE, A., SHARMAN, P., PERRY, M. & ADAMS, M. 2004. Increased apoptosis in human osteoarthritic cartilage corresponds to reduced cell density and expression of caspase-3. *Arthritis Rheum,* 50**,** 507-15.

SHINMEI, M., MASUDA, K., KIKUCHI, T., SHIMOMURA, Y. & OKADA, Y. 1991. Production of cytokines by chondrocytes and its role in proteoglycan degradation. *J Rheumatol Suppl,* 27**,** 89-91.

SHIZUME, K., LERNER, A. B. & FITZPATRICK, T. B. 1954. In vitro bioassay for the melanocyte stimulating hormone. *Endocrinology,* 54**,** 553-60.

SHLOPOV, B. V., GUMANOVSKAYA, M. L. & HASTY, K. A. 2000. Autocrine regulation of collagenase 3 (matrix metalloproteinase 13) during osteoarthritis. *Arthritis Rheum,* 43**,** 195-205.

SHLOPOV, B. V., LIE, W. R., MAINARDI, C. L., COLE, A. A., CHUBINSKAYA, S. & HASTY, K. A. 1997. Osteoarthritic lesions: involvement of three different collagenases. *Arthritis Rheum,* 40**,** 2065-74.

SHLOPOV, B. V., SMITH, G. N., JR., COLE, A. A. & HASTY, K. A. 1999. Differential patterns of response to doxycycline and transforming growth factor beta1 in the down-regulation of collagenases in osteoarthritic and normal human chondrocytes. *Arthritis Rheum,* 42**,** 719-27.

SINGH, G., MILLER, J. D., LEE, F. H., PETTITT, D. & RUSSELL, M. W. 2002. Prevalence of cardiovascular disease risk factors among US adults with self-reported osteoarthritis: data from the Third National Health and Nutrition Examination Survey. *Am J Manag Care,* 8**,** S383-91.

SLOMINSKI, A., ERMAK, G. & MIHM, M. 1996. ACTH receptor, CYP11A1, CYP17 and CYP21A2 genes are expressed in skin. *J Clin Endocrinol Metab,* 81**,** 2746-9.

SMITH, C. J., SUN, D., HOEGLER, C., ROTH, B. S., ZHANG, X., ZHAO, G., XU, X. B., KOBARI, Y., PRITCHARD, K., JR., SESSA, W. C. & HINTZE, T. H. 1996. Reduced gene expression of vascular endothelial NO synthase and cyclooxygenase-1 in heart failure. *Circ Res,* 78**,** 58-64.

SOLOMON, L. 2001. Clinical Features of Osteoarthritis. *In: Kelly WN, Harris ED, Ruddy S, Sledge CB, eds. Textbook of Rheumatology.* 6th ed. Philadelphia: WB Saunders.

STAR, R. A., RAJORA, N., HUANG, J., STOCK, R. C., CATANIA, A. & LIPTON, J. M. 1995. Evidence of autocrine modulation of macrophage nitric oxide synthase by alpha-melanocyte-stimulating hormone. *Proc Natl Acad Sci U S A,* 92**,** 8016-20.

STELLER, H. 1995. Mechanisms and genes of cellular suicide. *Science,* 267**,** 1445-9.

STETLER-STEVENSON, M., MANSOOR, A., LIM, M., FUKUSHIMA, P., KEHRL, J., MARTI, G., PTASZYNSKI, K., WANG, J. & STETLER-STEVENSON, W. G. 1997. Expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in reactive and neoplastic lymphoid cells. *Blood,* 89**,** 1708-15.

STOCKWELL, R. A. 1971. The interrelationship of cell density and cartilage thickness in mammalian articular cartilage. *J Anat,* 109**,** 411-21.

STOCKWELL, R. A. 1991. Cartilage failure in osteoarthritis: relevance of normal structure and function. A review. *Clin Anat,* 4**,** 161-191.

STRIETER, R. M., KUNKEL, S. L., ELNER, V. M., MARTONYI, C. L., KOCH, A. E., POLVERINI, P. J. & ELNER, S. G. 1992. Interleukin-8. A corneal factor that induces neovascularization. *Am J Pathol,* 141**,** 1279-84.

TAHERZADEH, S., SHARMA, S., CHHAJLANI, V., GANTZ, I., RAJORA, N., DEMITRI, M. T., KELLY, L., ZHAO, H., ICHIYAMA, T., CATANIA, A. & LIPTON, J. M. 1999. alpha-MSH and its receptors in regulation of tumor necrosis factor-alpha production by human monocyte/macrophages. *Am J Physiol,* 276**,** R1289-94.

TAKAHASHI, H., TERANISHI, Y., NAKANISHI, S. & NUMA, S. 1981. Isolation and structural organization of the human corticotropin--beta-lipotropin precursor gene. *FEBS Lett,* 135**,** 97-102.

TAMBLYN, R., BERKSON, L., DAUPHINEE, W. D., GAYTON, D., GRAD, R., HUANG, A., ISAAC, L., MCLEOD, P. & SNELL, L. 1997. Unnecessary prescribing of NSAIDs and the management of NSAID-related gastropathy in medical practice. *Ann Intern Med,* 127**,** 429-38.

TANIGUCHI, T. 1988. Regulation of cytokine gene expression. *Annu Rev Immunol,* 6**,** 439-64.

TATRO, J. B. 1996. Receptor biology of the melanocortins, a family of neuroimmunomodulatory peptides. *Neuroimmunomodulation,* 3**,** 259-84.

TAYLOR, A. & NAMBA, K. 2001. In vitro induction of CD25+ CD4+ regulatory T cells by the neuropeptide alpha-melanocyte stimulating hormone (alpha-MSH). *Immunol Cell Biol,* 79**,** 358-67.

TCHETINA, E. V., DI BATTISTA, J. A., ZUKOR, D. J., ANTONIOU, J. & POOLE, A. R. 2007. Prostaglandin PGE2 at very low concentrations suppresses collagen cleavage in cultured human osteoarthritic articular cartilage: this involves a decrease in expression of proinflammatory genes, collagenases and COL10A1, a gene linked to chondrocyte hypertrophy. *Arthritis Res Ther,* 9**,** R75.

TCHETINA, E. V., SQUIRES, G. & POOLE, A. R. 2005. Increased type II collagen degradation and very early focal cartilage degeneration is associated with upregulation of chondrocyte differentiation related genes in early human articular cartilage lesions. *J Rheumatol,* 32**,** 876-86.

TERKELTAUB, R., ZACHARIAE, C., SANTORO, D., MARTIN, J., PEVERI, P. & MATSUSHIMA, K. 1991a. Monocyte-derived neutrophil chemotactic factor/interleukin-8 is a potential mediator of crystal-induced inflammation. *Arthritis Rheum,* 34**,** 894-903.

TERKELTAUB, R. A., DYER, C. A., MARTIN, J. & CURTISS, L. K. 1991b. Apolipoprotein (apo) E inhibits the capacity of monosodium urate crystals to stimulate neutrophils. Characterization of intraarticular apo E and demonstration of apo E binding to urate crystals in vivo. *J Clin Invest,* 87**,** 20-6.

THOMAS, W. D., ZHANG, X. D., FRANCO, A. V., NGUYEN, T. & HERSEY, P. 2000. TNF-related apoptosis-inducing ligand-induced apoptosis of melanoma is associated with changes in mitochondrial membrane potential and perinuclear clustering of mitochondria. *J Immunol,* 165**,** 5612-20.

THOMPSON, C. B. 1995. Apoptosis in the pathogenesis and treatment of disease. *Science,* 267**,** 1456-62.

THORNBERRY, N. A. & LAZEBNIK, Y. 1998. Caspases: enemies within. *Science,* 281**,** 1312-6.

TIAN, X. M., CHEN, S. Q., MORALES, T. I. & HASCALL, V. C. 1989. Biochemical and morphological studies of steady state and lipopolysaccaride treated bovine articular cartilage explant cultures. *Connect Tissue Res,* 19**,** 195-218.

TOWER, G. B., COON, C. I., BELGUISE, K., CHALBOS, D. & BRINCKERHOFF, C. E. 2003. Fra-1 targets the AP-1 site/2G single nucleotide polymorphism (ETS site) in the MMP-1 promoter. *Eur J Biochem,* 270**,** 4216-25.

TRAVERSO, V., CHRISTIAN, H. C., MORRIS, J. F. & BUCKINGHAM, J. C. 1999. Lipocortin 1 (annexin 1): a candidate paracrine agent localized in pituitary folliculo-stellate cells. *Endocrinology,* 140**,** 4311-9.

TROYER, H. 1982. Experimental models of osteoarthritis: a review. *Semin Arthritis Rheum,* 11**,** 362-74.

TURK, B. 2006. Targeting proteases: successes, failures and future prospects. *Nat Rev Drug Discov,* 5**,** 785-99.

UHLER, M. & HERBERT, E. 1983. Complete amino acid sequence of mouse pro-opiomelanocortin derived from the nucleotide sequence of pro-opiomelanocortin cDNA. *J Biol Chem,* 258**,** 257-61.

VAN DAMME, J., BUNNING, R. A., CONINGS, R., GRAHAM, R., RUSSELL, G. & OPDENAKKER, G. 1990. Characterization of granulocyte chemotactic activity from human cytokine-stimulated chondrocytes as interleukin 8. *Cytokine,* 2**,** 106-11.

VAN DER KORST, J. K., SKOLOFF, L. & MILLER, E. J. 1968. Senescent pigmentation of cartilage and degenerative joint disease. *Arch Pathol,* 86**,** 40-7.

VAN KIMMENADE, R. R., JANUZZI, J. L., JR., ELLINOR, P. T., SHARMA, U. C., BAKKER, J. A., LOW, A. F., MARTINEZ, A., CRIJNS, H. J., MACRAE, C.

A., MENHEERE, P. P. & PINTO, Y. M. 2006. Utility of amino-terminal pro-brain natriuretic peptide, galectin-3, and apelin for the evaluation of patients with acute heart failure. *J Am Coll Cardiol,* 48**,** 1217-24.

VAN VENROOIJ, W. J. & PRUIJN, G. J. 2008. An important step towards completing the rheumatoid arthritis cycle. *Arthritis Res Ther,* 10**,** 117.

VAN VENROOIJ, W. J., VAN BEERS, J. J. & PRUIJN, G. J. 2008. Anti-CCP Antibody, a Marker for the Early Detection of Rheumatoid Arthritis. *Ann N Y Acad Sci,* 1143**,** 268-85.

VAN VENROOIJ, W. J. & ZENDMAN, A. J. 2008. Anti-CCP2 antibodies: an overview and perspective of the diagnostic abilities of this serological marker for early rheumatoid arthritis. *Clin Rev Allergy Immunol,* 34**,** 36-9.

VANE, J. R. 1971. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat New Biol,* 231, 232-5.

VANE, J. R. 1976. Prostaglandins as mediators of inflammation. *Adv Prostaglandin Thromboxane Res,* 2**,** 791-801.

VANE, J. R. 2000. The fight against rheumatism: from willow bark to COX-1 sparing drugs. *J Physiol Pharmacol,* 51**,** 573-86.

VANE, J. R. & BOTTING, R. M. 1998a. Anti-inflammatory drugs and their mechanism of action. *Inflamm Res,* 47 Suppl 2**,** S78-87.

VANE, J. R. & BOTTING, R. M. 1998b. Mechanism of action of antiinflammatory drugs. *Int J Tissue React,* 20**,** 3-15.

VAYSSIERE, B. M., DUPONT, S., CHOQUART, A., PETIT, F., GARCIA, T., MARCHANDEAU, C., GRONEMEYER, H. & RESCHE-RIGON, M. 1997. Synthetic glucocorticoids that dissociate transactivation and AP-1 transrepression exhibit antiinflammatory activity in vivo. *Mol Endocrinol,* 11**,** 1245-55.

VILLIGER, P. M., TERKELTAUB, R. & LOTZ, M. 1992. Production of monocyte chemoattractant protein-1 by inflamed synovial tissue and cultured synoviocytes. *J Immunol,* 149, 722-7.

VINCENTI, M. P., WHITE, L. A., SCHROEN, D. J., BENBOW, U. & BRINCKERHOFF, C. E. 1996. Regulating expression of the gene for matrix metalloproteinase-1 (collagenase): mechanisms that control enzyme activity, transcription, and mRNA stability. *Crit Rev Eukaryot Gene Expr,* 6**,** 391-411.

VISSE, R. & NAGASE, H. 2003. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ Res,* 92**,** 827-39.

VOISEY, J. & VAN DAAL, A. 2002. Agouti: from mouse to man, from skin to fat. *Pigment Cell Res,* 15**,** 10-8.

VON EGGELKRAUT-GOTTANKA, R. & BECK-SICKINGER, A. G. 2004. Biosynthesis of peptide hormones derived from precursor sequences. *Curr Med Chem,* 11**,** 2651-65.

VON ZASTROW, M. & KOBILKA, B. K. 1992. Ligand-regulated internalization and recycling of human beta 2-adrenergic receptors between the plasma

membrane and endosomes containing transferrin receptors. *J Biol Chem,* 267**,** 3530-8.

WAGENER, F. A., VOLK, H. D., WILLIS, D., ABRAHAM, N. G., SOARES, M. P., ADEMA, G. J. & FIGDOR, C. G. 2003. Different faces of the heme-heme oxygenase system in inflammation. *Pharmacol Rev,* 55**,** 551-71.

WANG, P., ZHU, F. & KONSTANTOPOULOS, K. 2010a. Prostaglandin E2 induces interleukin-6 expression in human chondrocytes via cAMP/protein kinase A- and phosphatidylinositol 3-kinase-dependent NF-kappaB activation. *Am J Physiol Cell Physiol,* 298**,** C1445-56.

WANG, P., ZHU, F., LEE, N. H. & KONSTANTOPOULOS, K. 2010b. Shear-induced interleukin-6 synthesis in chondrocytes: roles of E prostanoid (EP) 2 and EP3 in cAMP/protein kinase A- and PI3-K/Akt-dependent NF-kappaB activation. *J Biol Chem,* 285**,** 24793-804.

WATANABE, H., YAMADA, Y. & KIMATA, K. 1998. Roles of aggrecan, a large chondroitin sulfate proteoglycan, in cartilage structure and function. *J Biochem,* 124**,** 687-93.

WESTACOTT, C. I., ATKINS, R. M., DIEPPE, P. A. & ELSON, C. J. 1994. Tumor necrosis factor-alpha receptor expression on chondrocytes isolated from human articular cartilage. *J Rheumatol,* 21**,** 1710-5.

WESTACOTT, C. I. & SHARIF, M. 1996. Cytokines in osteoarthritis: mediators or markers of joint destruction? *Semin Arthritis Rheum,* 25**,** 254-72.

WIKBERG, J. E., MUCENIECE, R., MANDRIKA, I., PRUSIS, P., LINDBLOM, J., POST, C. & SKOTTNER, A. 2000. New aspects on the melanocortins and their receptors. *Pharmacol Res,* 42**,** 393-420.

WILLIS, D. 1999. *Overview of HO-1 in inflammatory pathologies,* Basel; Boston; Berlin, Birkhauser.

WILSON, B. D., OLLMANN, M. M., KANG, L., STOFFEL, M., BELL, G. I. & BARSH, G. S. 1995. Structure and function of ASP, the human homolog of the mouse agouti gene. *Hum Mol Genet,* 4**,** 223-30.

WILSON, W., VAN DONKELAAR, C. C., VAN RIETBERGEN, B., ITO, K. & HUISKES, R. 2004. Stresses in the local collagen network of articular cartilage: a poroviscoelastic fibril-reinforced finite element study. *J Biomech,* 37**,** 357-66.

WONG, M., ZIRING, D., KORIN, Y., DESAI, S., KIM, S., LIN, J., GJERTSON, D., BRAUN, J., REED, E. & SINGH, R. R. 2008. TNFalpha blockade in human diseases: mechanisms and future directions. *Clin Immunol,* 126**,** 121-36.

WOOD, N. C., DICKENS, E., SYMONS, J. A. & DUFF, G. W. 1992. In situ hybridization of interleukin-1 in CD14-positive cells in rheumatoid arthritis. *Clin Immunol Immunopathol,* 62**,** 295-300.

XIA, Y., WIKBERG, J. E. & CHHAJLANI, V. 1995. Expression of melanocortin 1 receptor in periaqueductal gray matter. *Neuroreport,* 6**,** 2193-6.

YAN, S., ZHOU, C., LOU, X., XIAO, Z., ZHU, H., WANG, Q., WANG, Y., LU, N., HE, S., ZHAN, Q., LIU, S. & XU, N. 2009a. PTTG overexpression

promotes lymph node metastasis in human esophageal squamous cell carcinoma. *Cancer Res,* 69**,** 3283-90.

YAN, Y. P., LANG, B. T., VEMUGANTI, R. & DEMPSEY, R. J. 2009b. Galectin-3 mediates post-ischemic tissue remodeling. *Brain Res,* 1288**,** 116-24.

YANG, Y., HUTCHINSON, P. & MORAND, E. F. 1999. Inhibitory effect of annexin I on synovial inflammation in rat adjuvant arthritis. *Arthritis Rheum,* 42**,** 1538-44.

YANG, Y., LEECH, M., HUTCHINSON, P., HOLDSWORTH, S. R. & MORAND, E. F. 1997. Antiinflammatory effect of lipocortin 1 in experimental arthritis. *Inflammation,* 21**,** 583-96.

YOON, S. W., CHUN, J. S., SUNG, M. H., KIM, J. Y. & POO, H. 2008. alpha-MSH inhibits TNF-alpha-induced matrix metalloproteinase-13 expression by modulating p38 kinase and nuclear factor kappaB signaling in human chondrosarcoma HTB-94 cells. *Osteoarthritis Cartilage,* 16**,** 115-24.

ZACHARIAE, C. O., ANDERSON, A. O., THOMPSON, H. L., APPELLA, E., MANTOVANI, A., OPPENHEIM, J. J. & MATSUSHIMA, K. 1990. Properties of monocyte chemotactic and activating factor (MCAF) purified from a human fibrosarcoma cell line. *J Exp Med,* 171**,** 2177-82.

ZHANG, Q., HUI, W., LITHERLAND, G. J., BARTER, M. J., DAVIDSON, R., DARRAH, C., DONELL, S. T., CLARK, I. M., CAWSTON, T. E., ROBINSON, J. H., ROWAN, A. D. & YOUNG, D. A. 2008. Differential Toll-like receptor-dependent collagenase expression in chondrocytes. *Ann Rheum Dis,* 67**,** 1633-41.

ZHONG, Q., SRIDHAR, S., RUAN, L., DING, K. H., XIE, D., INSOGNA, K., KANG, B., XU, J., BOLLAG, R. J. & ISALES, C. M. 2005. Multiple melanocortin receptors are expressed in bone cells. *Bone,* 36**,** 820-31.

ZHU, F., WANG, P., KONTROGIANNI-KONSTANTOPOULOS, A. & KONSTANTOPOULOS, K. 2010. Prostaglandin (PG)D(2) and 15-deoxy-Delta(12,14)-PGJ(2), but not PGE(2), mediate shear-induced chondrocyte apoptosis via protein kinase A-dependent regulation of polo-like kinases. *Cell Death Differ,* 17**,** 1325-34.

ZINN, W. M. 1970. Reflections on degenerative hip disease. *Ann Phys Med,* 10**,** 209-17.

Appendix
Publications