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Application of IL-4 Transgene Expression in a Chondrocyte-Based 3D Model of Inflammatory Arthritis

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APPLICATION OF IL-4 TRANSGENE EXPRESSION IN A CHONDROCYTE-BASED 3D MODEL OF INFLAMMATORY ARTHRITIS

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This humble effort and fruit of my thoughts and studies is dedicated to

Holy Prophet ***Muhammad*** (*Sallallaho-alahi-wa-sallam)*

The Greatest of the Prophets

This work was done at the Institute of Immunology and Molecular Biology (IMB), Philippstrasse 13, D-10115, Free University Berlin, Germany under the kind supervision of Prof. Dr. Michael F.G. Schmidt.

SUMMARY

Osteoarthritis (OA) is an imperative ailment in humans as well as in veterinary species especially dogs. Dogs constitute a good biological system to study human diseases because they suffer many diseases analogous to that of humans including OA. Chondrocytes, being the sole cell type in cartilage, are the solitary target of cytokines and other mediators involved in pathogenesis of OA. Among these cytokines, IL-1β and TNFα are two master pro-inflammatory cytokines whereas IL-4 is a major player with anti-inflammatory properties. Taking into consideration the chondrocyte biology and the cytokine hierarchy in OA, the present study was aimed at characterization of chondrocytes to develop a model of inflammatory arthritis where the anti-inflammatory capacity of IL-4 was to be monitored.

Isolated chondrocytes were grown in monolayer (2D) and in alginate-based 3D culture systems. Several morphological, biochemical, and functional features of chondrocytes were examined by immunocytochemistry, western blot and quantitative real time PCR. Attempts were made to generate a chondrocyte cell line by using human telomerase reverse transcriptase. Canine recombinant cytokines (IL-1β and TNFα) were cloned and their purified proteins used as exogenous stimulants in chondrocyte cultures. The expression of typical inflammation markers such as pro-inflammatory cytokines (IL-1β, IL-6, IL-8, GM-CSF and TNFα), enzyme mediators (MMP-3, MMP-13, iNOS, COX-2) and their catabolites (NO, PGE_2) was measured. IL-4 was transfected in chondrocytes in both culture systems followed by stimulation with IL-1β and TNFα to study its anti-inflammatory activity. IL-4 protein was detected by Western blot, and an ELISA system was developed for its quantification.

Results show that chondrocyte rapidly lose their characteristic phenotype in serial monolayer culture. This de-differentiation could be reverted by encapsulation of cells in alginate beads. Immortalized chondrocytes were able to produce chondrocyte specific markers in 3D culture but further studies were hampered due to their non-responsiveness to cytokines. Cytokine stimulation of chondrocytes resulted in high expression of all inflammatory markers. Western blot results showed IL-4 as a 17 kDa protein in transduced chondrocytes. The detection level for IL-4 as measured by ELISA was found to be about 3 ng ml^{-1}. The IL-4 expressed in chondrocytes was found to be biologically active since it was capable of inhibiting the selected inflammatory markers in the transduced chondrocytes. Since high expression of STAT6 (signal transducer and activator of transcription 6) was seen almost exclusively in cells expressing IL-4, it is

therefore assumed that IL-4 exerts its anti-inflammatory effects through STAT6 signaling. The canine form of STAT6 was identified and partially cloned and sequenced. Avenues are open to further characterize this signal molecule.

These findings altogether provide a solid foundation to validate constructs with cytokine-responsive “intelligent” promoters, which were developed in this laboratory alongside experimental work for this thesis. Thus, using the 3D culture systems described here it was shown that only in the presence of inflammation, the expression of IL-4 is driven in a sophisticated and fine-tuned manner. Thus, combining the above 3D culture system with the application of regulatable delivery of transgenes such as IL-4 or other promising candidate genes will facilitate further development and testing of novel gene therapy strategies against OA (and RA).

ZUSAMMENFASSUNG

Osteoarthritis (OA) ist eine ernste Erkrankung beim Menschen wie auch bei veterinärmedizinisch relevanten Säugetieren. Hunde stellen ein gutes biologisches System zur Untersuchung menschlicher Krankheiten dar, weil diese vielfach analog zu denen beim Menschen verlaufen. Dies gilt insbesondere auch für die OA. Chondrozyten sind als alleiniger Zelltyp des Knorpels und das Ziel von Zytokinen und anderen Mediatoren, die bei der Pathogenese der Arthrose auf dies Gewebe wirken. IL-1β und TNFα stellen die „Hauptakteure" unter den pro-inflammatorischen Zytokinen dar während IL-4 als Gegenspieler mit entzündungshemmenden Eigenschaften angesehen wird. Unter Berücksichtigung der Chondrozyten Biologie sowie der Hierarchie der Zytokine im Kontext der OA, wurde in der vorliegenden Studie die Charakterisierung caniner Chondrozyten vorgenommen. Ziel der Studie war, ein zellkulturbasiertes Modellsystem der inflammatorischen Arthritis zu entwickeln, mit dessen Hilfe die anti-inflammatorische Kapazität des IL-4 untersucht werden sollte.

Isolierte Chondrozyten wurden in Monolayer Kulturen (2-D) gezüchtet und in ein Alginat-basiertes dreidimensionales (3-D) Gewebesystem überführt. Eine Anzahl verschiedener morphologischer, biochemischer und funktioneller Parameter dieser vom Hund stammenden Chondrozyten wurde mit Hilfe der Immunzytochemie, des Western-Blots und der quantitativen Real-Time-PCR gemessen. Es wurde ein erfogreicher Versuch unternommen, unter Verwendung der klonierten menschlichen Telomerase-Reverse-Transkriptase eine (immortalisierte) canine Chondrozyten-Zelllinie zu etablieren. Zur Verwendung im angestrebten *in vitro* Entzündungsmodell wurden die Zytokine IL-1β und TNFα des Hundes kloniert und sequenziert, in *E. coli* exprimiert, aufgereinigt und charakterisiert.

Die Ausprägung der typischen Entzündungsmarker wie pro-inflammatorische Zytokine (IL-1β, IL-6, IL-8, GM-CSF, TNFα), Enzyme des Entzündungsgeschehens (MMP-3, MMP-13, iNOS, COX-2) und deren Kataboliten (NO, PGE_2) wurden gemessen. IL-4 wurde in mit IL-1β und TNFα stimulierten und transfizierten, Chondrozyten in beiden Kultursystemen (2-D und 3-D) auf seine anti-entzündliche Aktivität hin untersucht. Das in diesen caninen Zellkulturen exprimierte IL-4-Protein wurde nach PAGE von Lysaten im Western-Blot nachgewiesen und ein ELISA-System zu dessen Quantifizierung eingesetzt. Die Ergebnisse zeigen, dass Chondrozyten in der Monolayerkultur schnell ihren charakteristischen Phänotyp

verlieren. Diese Entdifferenzierung konnte durch die Verkapselung der Zellen in Alginat-Perlen (3-D Kultur) wieder revertiert werden. Immortalisierte Chondrozyten waren in der 3D-Kultur ebenfalls in der Lage, Chondrozyten-spezifische Marker zu exprimieren. Die Verwendung dieser Chondrozyten Zelllinie als *in vitro* Entzündungsmodell gelang aber nicht, weil diese Zellen nicht mehr auf die Behandlung mit pro-inflammatorischen Zytokinen reagierten. Dieselbe Stimulation normaler Chondrozyten führte dagegen zu einer hohen Expression aller ausgewählten Entzündungsmarker. Western-Blot-Ergebnisse zeigten, dass IL-4 in transduzierten Chondrozyten als 17 kDa-Protein vorliegt. Die im ELISA ermittelte Nachweisgrenze für IL-4 beträgt etwa 3 ng ml^{-1}. Das in transduzierten Chondrozyten exprimierte IL-4 erwies sich als biologisch aktiv, denn es bewirkte eine starke Hemmung der ausgewählten Entzündungsmarker.

Da ausschließlich in den Zellen, die IL-4 exprimieren, auch eine hohe Expression von STAT6 (Signal Transducer und Aktivator der Transkription 6) beobachtet wurde, wird angenommen, dass IL-4 seine anti-entzündliche Wirkung über den Signalweg des STAT6 entfaltet. In diesem Zusammenhang wurde die canine Form des STAT6 identifiziert, teilweise kloniert und sequenziert. Die weitere Charakterisierung dieses Signalproteins ist in unserem Labor im Gange.

Insgesamt liefern diese Erkenntnisse ein solides Fundament zur Validierung von neuartigen Expressionsvektoren mit zytokinsensitiven, „intelligenten“ Promotoren, die parallel zu den experimentellen Arbeiten für diese Dissertation entwickelt wurden. So konnte mit dem hier beschriebenen 3D-Kultur Systeme bereits gezeigt werden, dass bei Verwendung solcher Konstrukte die Expression von IL-4 nur in einem Entzündungsmilieu erfolgt. Die Kombination des oben genannten 3D-Kultur-Systems caniner Chondrozyten mit der Anwendung der entzündungsregulierten Expression von therapeutischen Transgenen wie IL-4 oder anderen Kandidaten-Gene kann für die Entwicklung und Testung neuer Gen-Therapie-Strategien gegen OA (und RA) nützlich sein.

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Finally I hope that the readers will forgive inadvertent errors.

Muhammad Farooq Rai

ABBREVIATIONS

µg	Micro gram
µl	Micro liter
µmol	Micro mole
2D	Two-dimensional
3D	Three-dimensional
ACLT	Anterior cruciate ligament transection
ACT	Autologous chondrocyte transplantation
APS	Ammonium Persulfate
BLAST	Basic local alignment and search tool
BMP	Bone morphogenic protein
bp	Base pairs
BSA	Bovine serum albumin
CBB	Coomassie brilliant blue
cDNA	Complementary DNA
CI	Type I collagen
CIA	Collagen induced arthritis
CII	Type II collagen
CLS	Chemiluminescence
CMV	Cytomegalovirus
COMP	Cartilage oligomeric matrix protein
Cox-2	Cyclooxigenase-2
CRTAC-1	Cartilage acidic protein-1
C_t	Cycle threshold
CTAB	Cetyl trimethylammonium bromide
DAB	Diaminobenzidine
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	2´-deoxynucleoside 5´-triphosphate
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme linked Immunosorbent assay
FACS	Fluorescence-activated cell sorter
FCS	Fetal calf serum
FS	Forward light scatter
GAPDH	Glycerinaldehyd -3–Phoshatdehydrognase
GD	Great Dane
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
h	Hour(s)
HRPO	Horseradish peroxidase
hTERT	Human telomerase reverse transcriptase
IFN–γ	Interferon gamma
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JNK	c-Jun-N-terminal kinase
kDa	Kilo-Dalton
L (l)	Liter
LAL	Limulus amoebocyte lysate
LD	Labrador
LPS	Lipopolysaccharide
LSAB	Labeled streptavidin biotin
min	Minute(s)
MMP	Matrix metalloproteinase
mRNA	Messenger RNA

NCBI	National centre for Biotechnology information
NF-κB	Nucleofector-kappa B
ng	Nanogram
Ni-NTA	Nickle-nitrilotriacetic acid
NK cell	Natural killer cell
nm	Nano meter
NO	Nitric oxide
NSAIDs	Non-steroidal anti-inflammatory drugs
OA	Osteoarthritis
OD	Optical density
OPDA	Ortho-phenylenediamine
P	Passage
PA	Plasminogen activator
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGE2	Prostaglandin E2
PMN	Polymorphonuclear neutrophils
PMSF	Phenylmethylsulphonyl fluoride
PVDF	Polyvinylidene difluoride
qRT-PCR	Quantitative real time PCR
RA	Rheumatoid arthritis
ra	Receptor antagonist
RACE	Rapid amplification of cDNA end
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT	Room temperature
R-T	Russian Terrier
RT-PCR	Reverse transcription PCR
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec	Second(s)
Sox-9	Sex-determining region Y (SRY)-box 9
SP	Shar Pei
SS	Side light scatter
STAT6	Signal transducer and activator of transcription 6
STET	Sucrose-Triton X-100-EDTA-Tris
TACE	TNFα converting enzyme
TAE	Tris-acetate-EDTA
TBS	Tris-buffered saline
TBST	TBS with Tween 20
TE	Tris-EDTA
TEMED	N,N,N´,N´ Tetramethyl-ethylenediamine
TGF	Transforming growth factor
Th cell	T helper cell
TIMP	Tissue inhibitor of metalloproteinases
TNF	Tumour necrosis factor
TRAP	Telomerase repeat amplification protocol
TXA_2	Thromboxane A_2
UV	Ultraviolet
WW	Weimaraner Welpen
YT	Yeast extract tryptone

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1.0. INTRODUCTION

1.1. Osteoarthritis (OA)

OA is a gradually progressing disorder of arthrodial joints of mammals, characterized by the destruction of articular cartilage, which results in discomfort and dysfunction of the affected joint. Its impact, upon both the patients, and the health-care systems, is difficult to overstate (Brooks, 2002). This disease is thought to originate from a series of complex interactions of both biochemical and biomechanical factors that occur simultaneously resulting in the breakdown of the cartilage matrix (Johnston, 1997).

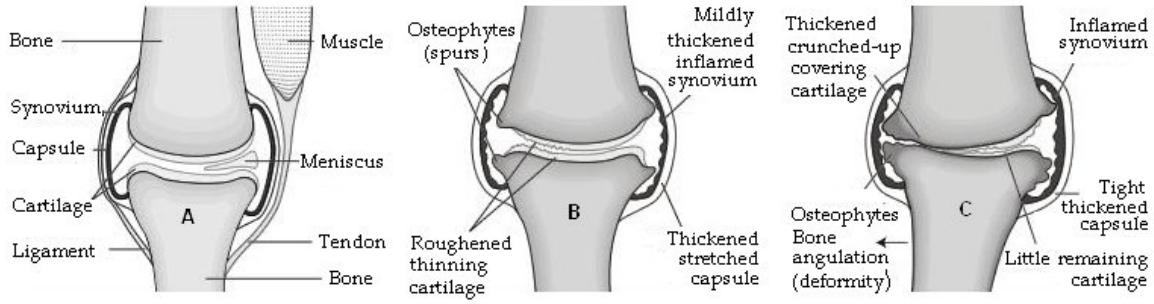

Fig. 1: Schematic representation of a normal joint and joints with mild and severe OA. (A) The normal joint with a thin layer of smooth, slippery cartilage. The joint is surrounded by synovium *which* has a tough outer layer called the capsule. The bones in the joint are kept firmly by the thick strong bands *called* ligaments which run within or just outside the capsule. The tendons are strong guiders that attach the muscles to the bones. They also help to keep the joint in place. (B) When a joint develops OA, the cartilage gradually roughens and becomes thin. The bone underneath thickens and grows outwards at the edge of the joint (this forms osteophytes or bony spurs). The synovium swells slightly and may produce extra fluid, which then makes the joint swell slightly. The capsule and ligaments slowly thicken and contract. Muscles that move the joint may weaken and become thin or wasted. (C) In severe OA, the cartilage can become so thin that it no longer covers the thickened bone ends. The bone ends touch and start to wear away. The loss of cartilage, the wearing of bone and the bony overgrowth at the edges can change the shape of the joint. This forces the bones out of their normal position and causes deformity. Figure downloaded from web (http://www.arc.org.uk/arthinfo/patpubs/6025/6025.asp).

Although biological and morphological changes initiated in OA are not constrained to articular cartilage, research has concentrated predominately on the pathogenesis of articular cartilage destruction. Primary pathways responsible for the disturbance in the balance in degradation and repair of the cartilage matrix are believed to be mediated through cytokines and chemokines (Fernandes *et al*., 2002). Cytokine activities are associated with the functional alterations in synovial membrane, cartilage and subchondral bone and are produced spontaneously or subsequently

stimulated by cells in joint tissue. They originate primarily from the synovial membrane (Martel-Pelletier *et al*., 1999), which reflects the inflammatory changes recognized in this tissue (Bondeson *et al*., 2006).

1.2. Epidemiology

Many species are affected by OA and it has been estimated that about 12.1% of the human population aged 25 to 74 (years) is affected (Lawrence *et al*., 1998). Up to 20% of the canine population at large over 1 year of age is affected by this anomaly. Larger breeds of dogs are more susceptible with either a rupture of cranial cruciate ligament or a hip dysplasia or osteochondrosis leading to OA (Johnston, 1997; Martinez, 2000; McLaughlin, 2000).

1.3. Etiology

The exact etiology of OA is not clearly known and is thought to be caused by a multitude of factors involving complex interplay between mechanical, cellular, and biochemical forces (Mansell and Bailey 1998; Nuki, 1999). Injury due to mechanical stress is also involved during initial stages of the disease and biochemical and genetic factors are likely to contribute to the further progression. Potential factors that may identify the progression of OA include systemic factors (metabolic, hormonal, genetic, and related to age or sex), local biomechanical factors (such as mechanical workload), body mass index and acetabular dysplasia, and already existing osteoarthritic changes such as signs visible on radiograph, clinical symptoms, and signs of cartilage degradation.

1.4. Clinical signs and symptoms

The pathology of OA involves the whole joint in a disease process that includes focal and progressive hyaline articular cartilage loss with concomitant changes in the bone underneath the cartilage, including development of marginal outgrowths, osteophytes, and increased thickness of subchondral bone. Soft-tissue structures in and around the joint are also affected. These structures include synovium, which may show modest inflammatory infiltrates, ligaments, which are often lax and bridging muscles, which become weak. Over time, the joint may lose its normal shape and bits of bone or cartilage can break off and float inside the joint space, causing more pain and damage. Loss of articular cartilage along with meniscal damage leads to narrowing of joint space with severe lameness (Reginster *et al.,* 2001; Karachalios *et al.,* 2007).

1.5. Pathogenesis

The key events in the pathology of OA have not yet been completely established. However, it is believed that this is characterized by degeneration of articular cartilage and proliferation of subchondral bone with associated synovial inflammation. The balance between the synthesis and degradation of cartilage observed in healthy individuals is disturbed in OA patients. An enhanced breakdown of cartilage matrix and reduced synthesis of matrix components by articular chondrocytes eventually leads to the destruction of the affected tissue.

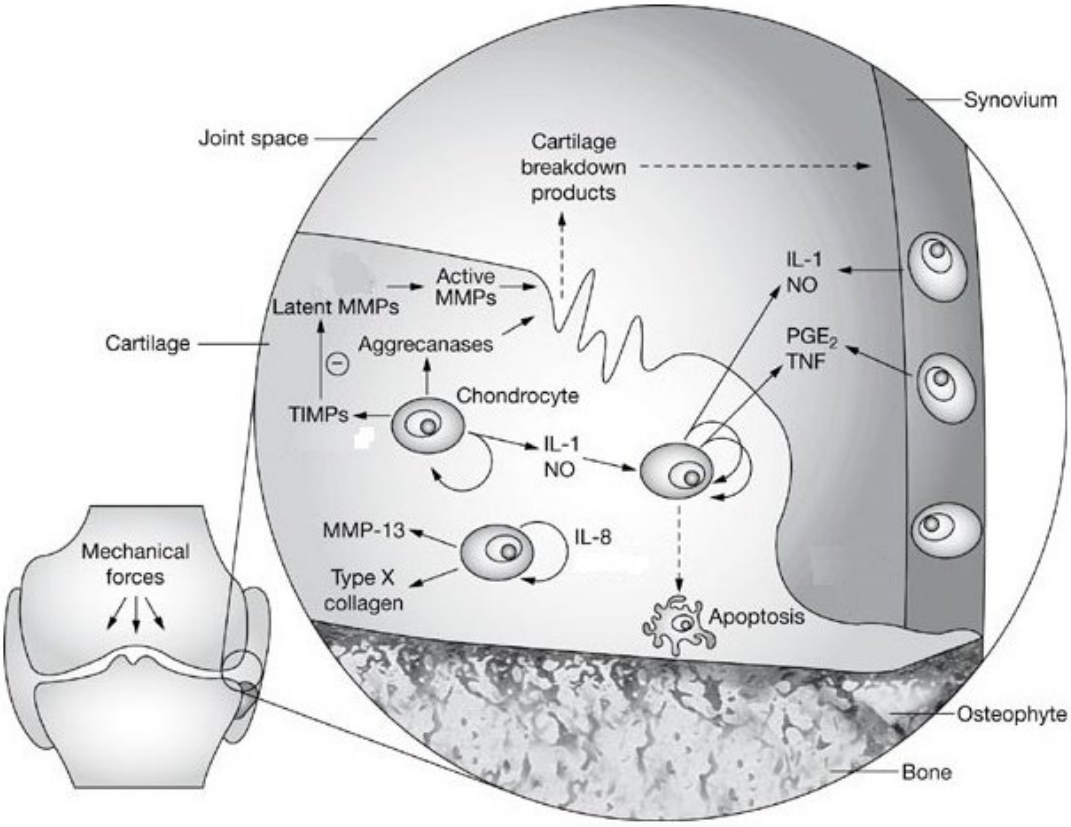

Fig. 2: Schematic representation of key pathological events and potential targets for disease modification in OA. Mediators that represent potential therapeutic targets have been identified in both synovial tissue and cartilage. Less well identified are targets derived from bone. BMP, bone morphogenic protein; IL, interleukin; MMP, matrix metalloproteinase; NO, nitric oxide; PA, plasminogen activator; PGE_2, prostaglandin E_2; TGF, transforming growth factor; TIMP, tissue inhibitor of metalloproteinase; TNF, tumor necrosis factor. Figure reproduced from Abramson *et al*. (2006).

An insight into the inflammatory pathway shows an imbalance between the Th_1 and Th_2 cells, which produce pro-inflammatory and anti-inflammatory cytokines, respectively (Liossis and Tsokos, 1998). The Th_1 cells secrete interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα), which are the prime candidates that trigger the inflammatory cascade (Fernandes *et al*., 2002) in OA. It is now well documented that

IL-1β and TNFα are the predominant pro-inflammatory and catabolic cytokines involved in disease initiation and progression. Other pro-inflammatory cytokines may amplify or modulate this process (Goldring, 2000a).

Moreover, the absence of IL-4, IL-10 and IL-13 which are secreted by Th_2 cells in the inflamed synovial fluid emphasizes that this disease is associated with an immunological imbalance. Both IL-1β and TNFα are synthesized as precursor proteins and are cleaved into active proteins by their respective converting enzymes.

These cytokines are believed to be synthesized by inflamed synovial membrane and diffuse into the articular cartilage. Subsequently, they activate signaling intermediates such as nuclear factor kappa B (NF-κB) and c-Jun N-terminal kinase (JNK) to initiate the transcription of downstream inflammatory genes like matrix metalloproteinases (MMPs), nitric oxide (NO), and also self propagation of IL-1β, TNFα, and other inflammatory cytokines like IL-2, IL-6, and IL-8 (Vincenti and Brinckerhoff, 2002). IL-1β and TNFα aid in progression of disease while MMPs and NO mediate the cartilage catabolism (Amin *et al.,* 2000). For the role of chondrocytes in these processes please see below.

1.6. Role of cytokines in OA

The already existing preliminary scientific results have been highly suggestive of the idea that cytokines play an essential role in the pathogenesis of OA. Nevertheless, the extent to which cytokines participate in the origin of OA, or are taken as the consequence of the OA process remains unanswered question (Steinmeyer, 2004).

Cytokines are closely associated with functional alterations in synovium, cartilage and subchondral bone, and are produced both spontaneously and following stimulation by the joint tissue cells. They appear to be first produced by the synovial membrane, and then diffuse into the cartilage through the synovial fluid. They activate the chondrocytes, which in turn produce catabolic factors such as proteases and multiple pro-inflammatory cytokines.

In a crude schematic manner, cytokines are subdivided into groups of pro-inflammatory and anti-inflammatory cytokines, whereby the two systems are held in equilibrium. With OA, current thinking assumes that a faulty homeostasis exists between the pro-inflammatory and the anti-inflammatory cytokines (Steinmeyer, 2004). Studies examining the contribution of cytokines to the pathogenesis of arthritic diseases, including OA, have focused mainly on pro-inflammatory cytokines such as

IL-1β, IL-6, IL-8, and TNFα and anti-inflammatory cytokines IL-4, IL-10, and IL-13 (Goldring *et al*., 2004; Hegemann *et al*., 2005; Maccoux *et al.,* 2007).

1.6.1. Pro-inflammatory cytokines

Pro-inflammatory cytokines are believed to play a pivotal role in the initiation and development of OA disease process and an excess of pro-inflammatory cytokines is thought to be responsible for its clinical manifestations. Other cytokines having pro-inflammatory properties or catabolic factors could also contribute to this pathological condition.

It is tempting to believe that IL-1β is a major catabolic cytokine with its crucial destructive role in chronic arthritis. In articular joint tissue, including synovial membrane, synovial fluid and cartilage, IL-1β has been found in the active form, and *ex vivo* experiments have demonstrated the ability of OA synovial membrane to secrete this cytokine (Pelletier *et al*., 1995). It is a key mediator of inflammation, tissue injury, and immunologic reactions. The biological activation of cells by IL-1β is mediated through association with specific cell-surface receptors. IL-1β induces chondrocytes and synovial cells to produce catabolic proteases and stimulates the production of prostaglandin E_2 (PGE_2) and pro-inflammatory cytokines such as IL-6 and IL-8 (Martel-Pelletier *et al*., 1999).

TNFα also appears to be an important mediator of matrix degradation and a pivotal cytokine in synovial membrane inflammation, although this cytokine is detected in OA articular tissue at a low level. TNFα induces multiple biological activities, and several distinct mechanisms of signal transduction may explain this diversity of action.

There is now considerable evidence to suggest that IL-1β and TNFα are the main mediators in the pathogenesis of OA. However, they may not play equivalent roles in animal models of arthritis. Yet it is claimed, and substantiated by studies on animal models, that IL-1β is of pivotal importance in cartilage destruction and TNFα appears to drive the inflammatory process (Van de Loo *et al*., 1995; Caron *et al*., 1996; Wagner *et al*., 1997; Martel-Pelletier, 1999). Both can induce chondrocytes and synovial cells, to produce other cytokines like IL-6, IL-8 and their own production, as well as stimulate proteases and PGE_2 production. Both of them play a direct role in altering normal cartilage and bone metabolism because each can induce collagenase expression in synovial cells, inhibit proteoglycan synthesis in articular chondrocytes, and stimulate bone resorption *in vitro* (Bertolini *et al*., 1986; Saklatvala, 1986).

IL-6 is a multifunctional cytokine with activities on immune cell function, proliferation, and differentiation (Hirano and Kishimoto, 1990). Both IL-1β and TNFα induce the synthesis of IL-6 and it has been shown that in arthritis models the production of TNFα precedes that of IL-6 (Wong and Clark, 1988). IL-6 is a potent stimulator of osteoblast-like cell development and induction of IL-6 in synovial fluid may contribute to damages to the cartilage matrix in joints (Lorenzo, 1991). This cytokine has also been proposed as a contributor to the OA pathological process by increasing the number of inflammatory cells in synovial tissue (Guerne *et al*., 1989), stimulating the proliferation of chondrocytes, and inducing an amplification of the IL-1β effects on the increased synthesis of MMPs and inhibiting proteoglycan production (Nietfeld *et al*., 1990).

IL-8 is a potent chemotactic cytokine for polymorphonuclear neutrophils (PMN), stimulating their chemotaxis and generating reactive oxygen metabolites (Yu *et al*., 1994). This chemokine is synthesized by a variety of cells including monocytes/macrophages, chondrocytes and fibroblasts (Koch *et al*., 1991; Kristensen *et al*., 1991). It has been reported that the strongest expression of IL-8, in both OA and rheumatoid arthritis (RA) patients, was detected in the blood vessels and lining cell layers of the resected synovial membrane (Deleuran *et al*., 1994). Researchers have shown that human articular chondrocytes, stimulated by IL-1β during response to cartilage injury, express and secrete bioactive IL-8 (Lotz *et al*., 1992). TNFα can stimulate the release of IL-8 by these cells (Hirota *et al*., 1992), and it is possible that IL-8 plays a role in the acute inflammatory reaction.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) was initially discovered for its role in the differentiation of hemopoietic precursor cells into mature granulocytes and macrophages (Metcalf, 1989); however, it can also affect mature cell function and may be considered pro-inflammatory (Hamilton *et al*., 1980; Alvaro-Gracia *et al.,* 1989). There is some evidence to suggest that GM-CSF has an influence on the pathogenesis of RA (Xu *et al*., 1989; de Vries *et al.,* 1991). GM-CSF is a potent accelerator of the pathological events leading to chronic inflammatory polyarthritis in murine collagen induced arthritis (CIA) supporting the notion that it may play a part in inflammatory polyarthritis, such as RA (Field and Clinton, 1993). GM-CSF receptor mRNA expression has been observed in both rheumatoid and osteoarthritic synovial cells by polymerase chain reaction analysis (Berenbaum *et al.,* 1994).

It is now evident that an activation of the cytokine cascades is involved in OA. However, apart from IL-1β and TNFα, the exact role of other pro-inflammatory cytokines has not been clearly established. Also, it has not yet been determined if they act independently or in concert, or whether a functional hierarchy exists between them (Martel-Pelletier, 1999).

1.6.2. Anti-inflammatory cytokines

Three anti-inflammatory cytokines (IL-4, IL-10, and IL-13) have been shown to be spontaneously elaborated by synovial membrane and cartilage. The anti-inflammatory properties of these cytokines include decreased production of IL-1β, TNFα, and MMPs, up-regulation of IL-1 receptor antagonist (IL-1ra) and tissue inhibitor of metalloproteinase-1 (TIMP-1), and inhibition of PGE_2 release (Donnelly *et al*., 1990; Vannier *et al*., 1992; Hart *et al*., 1995; Jovanovic *et al*., 1998; Alaaeddine *et al*., 1999). Although these cytokines share biological activities, their effects depend on the target cell of interest.

IL-4 has potent anti-inflammatory effects. The anti-inflammatory ability of IL-4 arises from its capacity to down-regulate the production of pro-inflammatory and Th_1 cytokines by inducing mRNA degradation and up-regulating expression of inhibitors of pro-inflammatory cytokines. Reduction of Th_1 cytokines results in suppression of both macrophage activation and production of IL-1β, IL-6, IL-8 and TNFα (Chomarat and Banchereau, 1997). IL-4 is a potent inhibitor of articular damage induced by IL-1β and TNFα (Yeh *et al*., 1995) by reducing synthesis of pro-inflammatory cytokines by up-regulating cytokine inhibitors and scavenging receptors (van Roon *et al*., 1996; Isömaki and Punnonen, 1997). The synthesis of NO is under the reciprocal regulation of interferon-γ (IFN-γ) and IL-4, where IL-4 inhibits IFN-γ induced release of NO from macrophages by down-regulating the activity of NO synthase (Sands *et al*., 1994). Since IL-1β and TNFα induce the synthesis of NO by synovial fibroblasts (Farrell *et al*., 1992) the IL-4-induced inhibition of NO is mediated by inhibiting the production of these cytokines. Furthermore, IL-4 inhibits the production of MMPs by macrophages, monocytes and fibroblasts (Corcoran *et al*., 1992; Prontera *et al*., 1996) and promotes the production of TIMP by chondrocytes (Shingu *et al*., 1995). IL-4 has also been shown to suppress TNFα-mediated PGE_2 production by OA synovial fibroblasts (Alaaeddine *et al*., 1999). In a recent study, it has been shown that an IL-4 gene transferred intra-articularly to rats with adjuvant induced arthritis significantly reduced synovial vessel density and ankle joint inflammation (Haas *et al*, 2006). The influence

of pro-inflammatory cytokines and subsequently the possible beneficial role of IL-4 on chondrocyte viability (necrosis/apoptosis), proliferation and NO production have been investigated and it was found that IL-4 can inhibit the effect of IL-1β and TNFα on NO production and proliferation of chondrocytes (Schuerwegh *et al*., 2003). The anti-inflammatory activity of canine IL-4 in chondrocytes is mediated through repression of nuclear factor kappa B or NF-κB (Ohmori and Hamilton, 2000) which is central to the pathogenesis of OA/RA and the associated inflammation. It is reported that that IL-4 activates STAT6 and the novel mechanism for IL-4 mediated inhibition of inflammatory gene expression provides an example of a STAT6 involvement (Bennett *et al.,* 1997).

IL-10 is a cytokine with potent anti-inflammatory properties and the capacity to down-regulate IL-1β and TNFα *in vitro* (Fernandes *et al*., 2002). It was found that IL-10 modulated TNFα production by increasing the release of the TNF soluble receptor from monocytes in culture, while down-regulating the receptor surface expression (Hart *et al*., 1995).

IL-13 inhibits TNFα production by mononuclear cells from peripheral blood and in OA synovial fibroblasts. (de Waal Malefyt et al., 1993). IL-13 has been shown to have important biological activities such as inhibiting the production of a wide range of pro-inflammatory cytokines in monocytes/macrophages, B cells, NK cells and endothelial cells, while increasing IL-1ra production (de Waal Malefyt et al., 1993; Defrance et al., 1994).

1.7. Role of enzyme mediators in OA

1.7.1. Matrix metalloproteinases (MMPs)

MMPs derived from chondrocytes, synovium, and polymorphonuclear leukocytes play a major role in pathological cartilage degradation seen in OA (Murphy *et al*., 1990; Woessner and Gunja-Smith, 1991) and mediate matrix degradation and subsequent joint destruction (Mengshol *et al*., 2002). The expression of inducible MMPs (MMPs 1, 3, 9, 13) in cartilage is stimulated by inflammatory cytokines, such as IL-1β and TNFα. In contrast to RA, OA synovium and synovial fluid contain markedly low levels of MMPs, and expression is restricted to active OA cartilage lesions, where destruction of type II collagen and proteoglycans occurs (Mitchell *et al*., 1996; Freemont *et al*., 1997; Shlopov *et al*., 1997). The fragments remaining after the initial cleavage by collagenases become susceptible to degradation by other enzymes, such as MMP-2, MMP-9, MMP-3, and cathepsin B. Of the three collagenases MMP-

13 has assumed greater importance in OA because it preferentially degrades type II collagen (Knäuper *et al*., 1996), and its expression is markedly increased in OA (Reboul *et al*., 1996; Shlopov *et al*., 1997; Tetlow *et al*., 2001). Studies have shown that the enhanced cleavage of type II collagen in cultured OA cartilage samples is arrested in the presence of a selective collagenase inhibitor against MMP-13 and MMP-8 (Dahlberg *et al*., 2000). Enhanced levels of MMP-3 and MMP-13 have been consistently identified in OA cartilage in various animal models. It is comforting to see the demonstration of high MMP-3 levels in the cartilage of dogs with OA, secondary to naturally acquired cranial cruciate ligament rupture. The expression of MMP-13 in OA cartilage and its ability to more effectively degrade type II collagen suggest a critical role for this enzyme in cartilage degradation (Knäuper *et al*., 1996; Mitchell *et al*., 1996). MMP-3 production in cartilage explants culture appeared to correlate with OA severity (Spreng *et al*., 1999). Recent attention has focused on the enzymes responsible for the degradation of the aggrecan core protein. While MMP-3 has the capacity to degrade aggrecan (Fosang *et al*., 1994; Lark *et al*., 1997; Büttner *et al*., 1998), there is evidence that degradation at the aggrecanase cleavage site is the primary event in chondrocyte-mediated catabolism of aggrecan (Hughes *et al*., 1995; Lark *et al*., 1995).

1.7.2. Inducible nitric oxide synthase (iNOS)

iNOS was first described in activated macrophages (Hibbs *et al*., 1988). This isoform is not usually expressed in healthy quiescent cells, but is rapidly induced in multiple cell types (endothelial and epithelial cells, chondrocytes and synoviocytes) stimulated with bacterial endotoxins or pro-inflammatory cytokines (Vallance and Leiper, 2002). Its expression is regulated at the transcriptional level and at the level of iNOS mRNA stability (Kleinert *et al*., 2000). Activation of transcription factor NFκB seems to be an essential step for iNOS induction in most cells (Förstermann and Kleinert, 1995). Once induced, iNOS produces high amounts of NO for a prolonged period of time (Nathan, 1992).

1.7.3. Cyclooxygenase-2 (COX-2)

Two isoforms of cyclooxygenase (COX), COX-1 and COX-2, catalyze the rate-limiting step in the conversion of arachidonic acid to prostaglandins and thromboxane A_2 (TXA_2) (Xie *et al*., 1991; Smith *et al*., 1996). COX-1 is a constitutively expressed protein that is thought to produce basal concentrations of prostaglandins and TXA_2, both of which are considered necessary for normal physiologic functions in many

tissues (Vane, 1994). COX-2 is induced *ex vivo* by cytokines in synovial tissue fibroblasts isolated from RA patients and is thought to play a primary role in the pain and inflammation associated with that disease (Xu *et al*., 1997; Raisz, 1999). Various studies demonstrated high levels of COX-2 expression and PGE_2 production following *ex vivo* stimulation by IL-1β, in the synovial membrane as well as in other human OA tissues including the meniscus and osteophytic fibrocartilage. Such data indicate that these tissues are important sources of PGE_2 produced by COX-2, which may play a role in the degradation of adjacent articular cartilage. Furthermore, the enhanced COX-2-induced PGE_2 production in fibrocartilage suggests a role for PGE_2 in the secondary remodeling of tissue that results in the formation of osteophytes. Thus, besides mediating pain and inflammation in OA, COX-2-generated PGE_2 may play a role in bone and cartilage degeneration that is associated with this disease.

1.8. Role of inflammation in OA and inflammatory mediators

OA is characterized by deterioration of articular cartilage (through the action of degrading enzymes) and non-purulent inflammation (Johnston, 1997). The episodic inflammation at the clinical stage of OA is now a well documented phenomenon and believed to be involved in the disease progression. In OA, classical signs of inflammation are absent since there is no marked infiltration of inflammatory cells into joint tissues, and the synovial fluid usually contains only few neutrophils. Nevertheless, much evidence supports the view that inflammatory components are still centrally involved in the disease process (Hedbom and Häuselmann, 2002). Thus, signs and symptoms of inflammation, including joint pain, swelling and stiffness are frequently present not only in RA, but also in OA (Felson, 2006).

Contrary to RA, synovitis has been considered as a secondary phenomenon not associated with systemic inflammation but it is responsible for symptoms of OA. Although inflammation and the immune response are unlikely to initiate synovitis, growing evidence has shown that inflammation accelerates articular degeneration (Pelletier *et al*., 2001). Although there remains debate in rating the role of synovial inflammation in OA, synovitis involving infiltration of activated B cells and T-lymphocytes and over-expression of pro-inflammatory mediators is common in early and late OA (Benito *et al*., 2005). Synovial inflammation is a factor that likely contributes to dysregulation of chondrocyte function, favoring an imbalance between the catabolic and anabolic activities of the chondrocyte in remodeling the cartilage extracellular matrix (ECM) (Loeser, 2006).

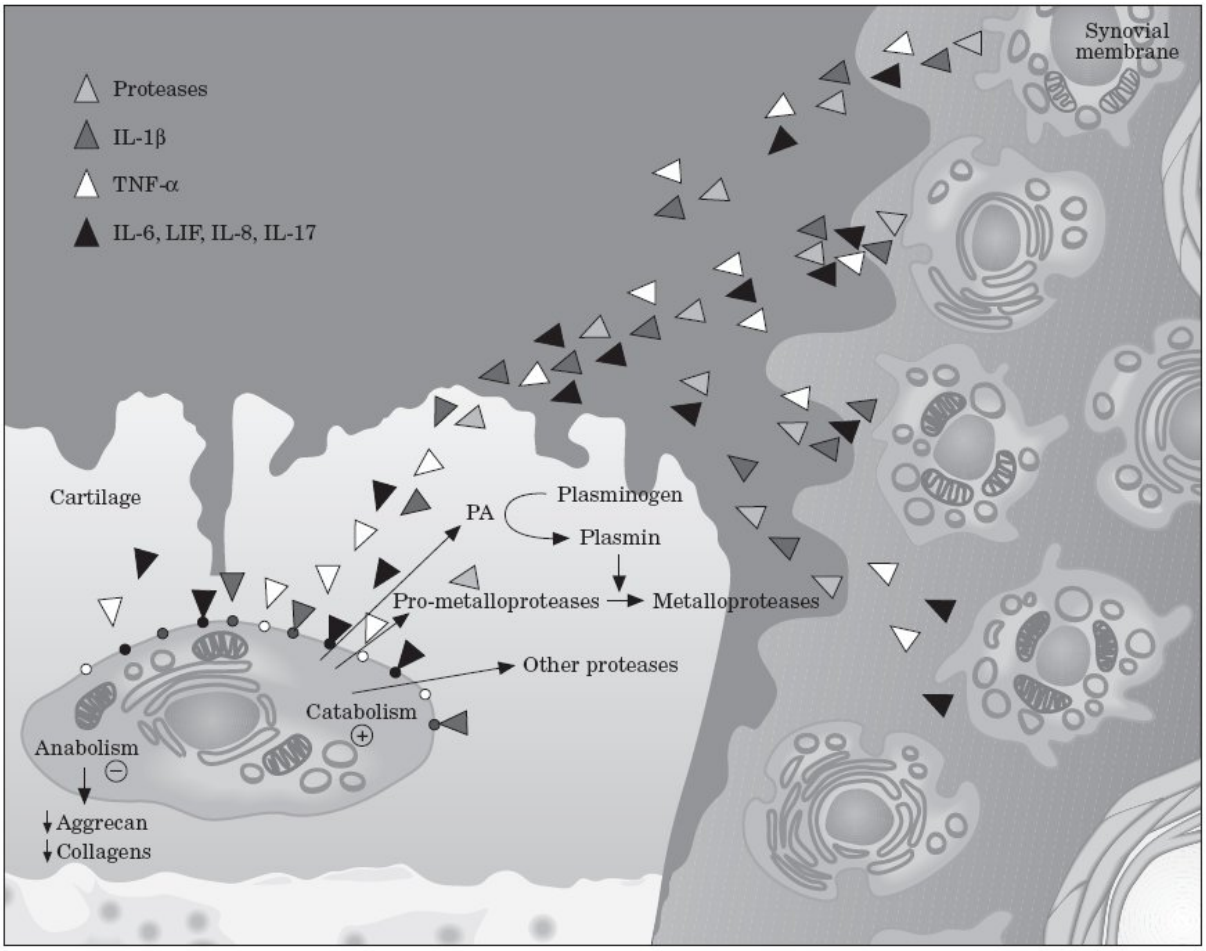

Fig. 3: Process involved in OA. At a later stage of the disease, inflammation of the synovial membrane occurs from which proteases and pro-inflammatory cytokines are released, and diffuse through the synovial fluid into the cartilage. This induces additional breakdown of cartilage matrix macromolecules. At this stage, the chondrocytes are hyper-responsive to cytokine stimulation because of an increased level of cytokine receptors on the cell membrane. Figure reproduced from Martel-Pelletier (1999).

Inflammatory mediators are generally agreed to induce cartilage degradation in OA. Various studies have drawn attention to two mediators that are locally produced at sites of inflammation: NO and PGE_2. These factors are released if isolated chondrocytes are stimulated with cytokines (Stadler *et al*., 1991; Palmer *et al*., 1993; Geng *et al*., 1995; Attur *et al*., 1997). The production of NO and PGE_2 is increased in human OA-affected cartilage. These and other inflammatory mediators are spontaneously released by OA cartilage explants *ex vivo*. The excessive production of NO inhibits matrix synthesis, and promotes its degradation. PGE_2 exerts both anabolic and catabolic effects on chondrocytes, depending on the microenvironment and physiological condition.

1.8.1. Nitric oxide (NO)

A role of NO in OA cartilage pathology and chondrocyte apoptosis has emerged from studies in anterior cruciate ligament transection (ACLT) models in rabbits and dogs (Hashimoto *et al*., 1999). Similar evidence of enhanced NO metabolite production was obtained in dogs with ligament rupture (Spreng *et al*., 2000). NO is a pleiotropic mediator in IL-1β-driven joint pathology that has been shown to be intimately involved in the OA catabolic process (Amin *et al*., 2000; Clancy *et al*., 2001; Nishida *et al*., 2001). One of the mechanisms by which cytokines elicit their effects involves the stimulation of NO production via iNOS (Stadler *et al*., 1991; Amin and Abramson, 1998; Martel-Pelletier *et al*., 1999). NO regulates chondrocyte and cartilage function in various ways. These include: 1) inhibition of the synthesis of cartilage-specific ECM molecules, such as type II collagen and proteoglycan by triggering de-differentiation of chondrocytes (Taskiran *et al*., 1994; Cao *et al*., 1997); 2) an increase in the number of apoptotic cells, which correlates with the extent of cartilage matrix loss (Blanco *et al*., 1998; Hashimoto *et al*., 1998); and 3) modulation of MMPs to cause degradation of cartilage matrix (Murrell *et al*., 1995). NO produced by iNOS regulates the functional activity, growth and death of many immune and inflammatory cell types including macrophages, T-lymphocytes, antigen-presenting cells, mast cells, neutrophils, and NK cells (Coleman, 2001).

1.8.2. Prostaglandin E_2 (PGE_2)

COX-2 and its major pro-inflammatory product, PGE_2, have been shown to be induced by IL-1β. Biochemical, genetic, and clinical evidence indicates that PGE_2 plays a critical role in inflammation and in the pathophysiology of articular joint diseases, such as RA and OA. For example, arthritic joint tissues produce large quantities of PGE_2 (Amin *et al*., 1997). More direct evidence for the role of PGE_2 in arthritis has been provided by gene targeting studies. Genetic disruption of either the PGE_2 receptor EP4 (McCoy *et al*., 2002) or COX-2 (Myers *et al*., 2000) reduced incidence and severity of collagen-induced arthritis in mice. These animals showed reduced inflammation and less cartilage and bone destruction. The role of PGE_2 in arthritis is also supported by effective suppression of pain and inflammatory responses in arthritis by NSAIDs that reduce PGE_2 biosynthesis (Crofford, 2002). Chondrocytes are a major source of PGE_2 in the joint; the production of this prostanoid can be induced by pro-inflammatory cytokines, mitogens, mechanical

stress, and trauma (Chrisman *et al*., 1981; Fermor *et al*., 2002; Martel-Pelletier *et al*., 2003).

1.9. Role of cartilage and chondrocytes in OA

1.9.1. Physiology of cartilage

Cartilage is a connective tissue that composes most of the skeleton of vertebrate embryos and, except for a small number of structures, is replaced by bone during ossification in the higher vertebrates. The three major types of cartilage that can be found in the mammalian body are hyaline cartilage, elastic cartilage, and fibrocartilage. The most prevalent, hyaline cartilage is a smooth and resilient material and is present at the articular surface of joints (Pritzker, 1998; López-Armada *et al*., 2004). It allows almost frictionless articulation and provides a medium by which load-bearing compressive and shearing stresses are uniformly dissipated across the joint (Mow and Ateshian, 1997; Ashkenazi and Dixit, 1998). It consists essentially of chondrocytes (2-5% from total tissue) and an ECM. The molecular composition of ECM is predominantly the fibrillar collagen, type II (5%), and the proteoglycan, aggrecan (20%). The chondrocytes are important in the control of matrix turnover through the production and secretion of collagen (mainly type II), proteoglycans, and enzymes for cartilage metabolism. The proteoglycans secreted into the matrix serve to trap and hold water in order to regulate the hydration of the matrix, and are responsible for the compressive strength (stiffness) of the cartilage tissue. Proteoglycans comprise proteoglycan aggrecan molecules, which are in turn made of basic subunits called glycosaminoglycans (Heinegård *et al*., 1998). Articular cartilage lacks blood vessels, lymphatics and nerves, therefore nutrients diffuse through the matrix from the surrounding synovial fluid. Being avascular, it has limited ability to repair itself and hence has higher risk of degeneration upon injury or with age (Setton *et al*., 1999). Collagen, proteoglycan and water interact to form a porous-permeable, fiber reinforced matrix that possesses the mechanical properties to sustain daily high stresses upon the articular cartilage (Andriacchi *et al*., 1997).

1.9.2. Cartilage/chondrocyte markers

Recently, autologous chondrocyte transplantation (ACT) has been introduced as a novel biological treatment (Brittberg *et al*., 1994). This approach, however, is hampered by its dependence of prior cultivation in order to increase the number of chondrocytes for transplantation. Consequently, the identification of the differentiation

or de-differentiation processes in chondrocytes is of major interest in any biological therapy approach.

1.9.2.1. Collagen

Collagen, a major component of the ECM of connective tissues, not only provides a structural support to these tissues but can also affect cell behaviour and gene expression. Collagen is the most abundant protein in the body with high structural organization and provides fibrous ultra structure to the tissues. Cartilage contains several different types of collagen – II, VI, IX, X, XI. Type II collagen accounts for 90-95% of collagen volume in normal articular cartilage and forms the primary component of cross banded fibrils (Mankin *et al*., 2000). The product of the type II collagen gene is an early and practically unique marker of chondrocyte differentiation. Type I collagen is a primary collagen in many tissues, including fibrocartilage, bone, tendon, and skin. In articular cartilage, it is produced early in chondrogenesis and becomes undetectable later during development (Morrison *et al*., 1996) and is found in remodeling and osteoarthritic cartilage (Miosge *et al*., 1998; Pei *et al*., 2000).

1.9.2.2. Aggrecan

Proteoglycan is a protein-polysaccharide molecule with glycosaminoglycan side chains (Fosang and Hardingham, 1996). The composition and concentration of proteoglycans is not the same throughout the cartilage. It changes with age, cartilage injury and disease (Setton *et al*., 1999). Aggrecan (large proteoglycans found in articular cartilage) is an approximately 200 nanometer long protein to which glycosaminoglycan chains and oligosaccharides are covalently attached (Muir, 1983; Fosang and Hardingham, 1996). Aggrecan molecules have a large number of chondroitin and keratin sulphate chains (Setton *et al*., 1999) and differ in length, molecular weight and composition. It is a characteristic proteoglycan produced by chondrocytes.

1.9.2.3. Cartilage acidic protein1 (CRTAC1)

In the search for new marker genes suitable to distinguish chondrocyte-like phenotype from osteoblasts and mesenchymal stem cells in culture, a novel gene called chondrocyte expressed protein-68 (CEP-68) has recently been identified and characterized. CEP-68 was then renamed as cartilage acidic protein 1 (CRTAC1). It harbours an N-terminal leader peptide and an epidermal growth factor-like calcium-

binding domain. CRTAC1 defines a new family of proteins and complements type II collagen as a new marker for stem-cell based chondrogenic tissue engineering (Steck *et al*., 2001). It will, therefore, be interesting to learn more about the possible function of this new potential matrix molecule (Benz *et al*., 2002). Quantitative gene expression of common cartilage and cell interaction molecules was analyzed using complementary DNA array technology and reverse transcription-polymerase chain reaction during optimization of cell differentiation of human stem cells, in order to achieve a molecular phenotype similar to that of chondrocytes in cartilage. It was found that CEP-68 was undetectable in monolayer chondrocytes compared with the healthy native cartilage (Winter *et al*., 2003).

1.9.2.4. Cartilage oligomeric matrix protein (COMP)

COMP is an oligomeric glycoprotein synthesized by chondrocytes in all types of cartilages. It is one of the main non-collagenous proteins of the ECM and can be found in articular cartilage (Hedbom *et al*., 1992). COMP is a sensitive marker for the differentiation state of articular primary chondrocytes and a novel phenotypical marker which is down-regulated faster than the widely accepted marker, collagen II. The different kinetics of COMP suggests differential regulation at the level of transcription (Zaucke *et al*., 2001).

1.9.2.5. Sex determining region Y- box 9 (Sox9)

Many lines of evidence, both *in vitro* and *in vivo* have shown that sex-determining region Y-type high mobility group box (Sox) proteins are necessary for chondrogenesis. Like other Sox proteins Sox9 binds to specific sequence in the minor groove of DNA and contains a potent transcription activation domain located at the carboxy-end of the protein, and hence is a typical transcription factor. It is involved in chondrocyte differentiation and maintenance of the chondrocytic phenotype (Zhao *et al*., 1997; Lefebvre and de Crombrugghe, 1998; Hering, 1999). The Sox9 gene is expressed in all chondroprogenitor cells in mouse embryos and at higher levels in chondrocytes but its expression is completely shut off in hypertrophic chondrocytes (Zhao *et al*., 1997). It is a transcriptional factor that is essential for chondrocyte differentiation (de Crombrugghe *et al.,* 2000), and the loss of chondrocyte differentiated characteristics *in vitro* has been linked to the reduced expression of chondrocyte-specific transcription factors, principally Sox9 (Lefebvre *et al*., 1998). Studies are in progress to identify other target genes for Sox9 in chondrocytes and also other transcription factors that are believed to cooperate with

Sox9 in the activation of chondrocyte-specific genes. Defining Sox9 function and the mechanisms that regulate Sox9 gene expression should contribute to a better understanding of chondrocyte differentiation (Lefebvre and de Crombrugghe, 1998).

To see whether Sox9 might control Col2a1 expression in chondrocytes, researchers showed that there is an excellent correlation between expression of type II and Sox9 in chondrogenic cells. It was found that Sox9 binds directly to the type II collagen enhancer at a site which is essential for chondrocyte-specific expression, and that forced expression of Sox9 is sufficient to activate this enhancer at high levels in non-chondrogenic cells. These results strongly suggest that Sox9 plays a crucial role in the specific activation of type II collagen in chondrocytes and raise the possibility that Sox9 might be a key transcriptional regulator of chondrocyte differentiation. The same authors revealed that Sox9 and type II collagen RNA levels decreased in parallel in chondrocytes during their progressive de-differentiation in culture (Lefebvre *et al*. 1997). Sox9 was shown to bind to and activate chondrocyte-specific enhancer elements in type II collagen and aggrecan *in vitro* (Bell *et al*., 1997; Lefebvre *et al*., 1997; Bridgewater *et al*., 1998; Xie *et al*., 1999; Liu *et al*., 2000; Sekiya *et al*., 2000; Zhang *et al*., 2003).

1.9.3. Role of chondrocytes in OA

Chondrocytes are highly specialized cells of mesenchymal origin that are responsible for laying down, sustaining, and degrading the extensive cartilage ECM, that functions as a shock absorber for the underlying bone. They are the only cell type present in adult hyaline cartilage, and they are important in the control of cartilage integrity (Aigner *et al*., 2001). Chondrocytes are terminally differentiated cells which despite their small number are responsible for the synthesis and maintenance of the entire tissue ECM (Fosang and Hardingham, 1996; Buckwalter and Mankin, 1997). The chondrocytes sense and respond to various mechanical stimuli through multiple regulatory pathways. This response can occur at multiple sites: the transcriptional (Valhmu *et al*., 1998) the translational or at the post-translational level (Kim *et al*., 1996). It may also involve changes in cell mediated assembly and matrix degradation mechanisms (Quinn *et al*., 1998). The metabolism and metabolic response of chondrocytes to external stimuli alter with aging. With age their ability to respond to anabolic stimuli and growth factors as well as their capacity to produce some types of proteoglycan decrease, and their proliferative capacity declines (Martin and Buckwalter, 2001; Martin and Buckwalter, 2002).

It has been proposed that OA is characterized by a process during which the chondrocytes are stimulated through mechanical events (use or trauma) or genetic assault (mutation of a matrix molecule), to respond by trying to remove and repair the damaged matrix (Fukui *et al*., 2001). Unfortunately these events result in a ‘’vicious circle’’ that ends when the balance of catabolic events outstrips the anabolic events, leading to rapid degeneration of the cartilage.

The biosynthetic studies reported in the literature strongly suggest that there is a change in the metabolism of chondrocytes that may affect the progression of OA. At this time, there has not been enough work done to sort out when and where the cells may be making the proper matrix for repair or whether some populations of chondrocytes can affect repairs better than others. The sequence of changes in the biosynthesis, however, indicates that there may be a difference in the synthesis of matrix components in OA cartilage as compared with normal cartilage. These differences could result in the laying down of matrix that is less structurally sound in OA cartilage as compared with normal cartilage, thus contributing to the perpetuation of degradation (Sandell, 2007).

The enhanced and coordinated expression of pro-inflammatory cytokines (IL-1β, TNFα) and inducible iNOS by chondrocytes strongly support the hypothesis that chondrocytes are the major site of production of inflammation mediators responsible for cartilage matrix degradation in OA, thus playing a primary role in the pathogenesis of this disease (Goldring, 2000b).

1.9.4. Cartilage degradation in OA

Although the etiology remains unclear, degradation of cartilage matrix components is generally agreed to be due to an increased synthesis and activation of extracellular proteinases, mainly MMPs. Up-regulation of proteinase activities has been implicated in the disease as the major cause of increased matrix catabolism. Degradation of structural macromolecules like proteoglycans and collagens leads to depletion of the most important building blocks of the ECM. Insufficient synthesis of new matrix macromolecules is also thought to be involved, possibly as a consequence of deficient stimulation by growth factors (Hedbom and Häuselmann, 2002).

Although OA is defined as a non-inflammatory arthropathy, considerable data implicate a role for pro-inflammatory cytokines derived from both the synovium and the chondrocytes themselves in the cartilage destruction associated with OA. Among such pro-inflammatory cytokines, IL-1β and TNFα have been implicated as important

mediators in the disease process (Goldring, 2000a). In response to these cytokines, chondrocytes up-regulate the production of NO and PGE_2, two factors that have been shown to induce a number of the cellular changes associated with OA. The generation of these key signal molecules depends on inducible enzymes and can be suppressed by pharmacological inhibitors.

Mechanical stress or injury may provide the initial stimulus for the cascade leading to cartilage degradation. Chondrocyte stimulation also leads to an increase in enzymes that are capable of degrading ECM components (Hollander *et al*., 1995).

As the exact molecular sequence of events is unknown, the degenerative process in OA progresses slowly over many years. Evidence suggests that cytokines produced by both synovial cells and chondrocytes, are key mediators of joint destruction in OA (Martel-Pelletier *et al*., 1992; Schlaak *et al*., 1996; Westacott and Sharif, 1996; Chevalier, 1997; Pelletier *et al*., 1997; Smith *et al*., 1997; Towle *et al*., 1997; Attur *et al*., 1998). In the normal articular joint, cartilage homeostasis is maintained by a balance between cytokine-mediated anabolic and catabolic processes. However, in OA, the balance shifts toward catabolism, leading to cartilage destruction and this catabolic cascade involves many pro-inflammatory mediators and enzymes.

Cartilage ECM degradation and suppression of chondrocyte ECM biosynthesis is regulated by a significant increase in the levels of cytokines in the affected synovial joint. While OA is considered a ‘’non-inflammatory arthropathy’’ recent compelling evidence indicates that increased cytokine production in OA is observed in all three joint compartments: synovial membrane, cartilage and subchondral bone (Malemud, 2004). The initiating factors are not well understood, nor are the relative contributions to the joint damage in individual patients at a specific stage of disease from each compartment. Central to the disordered metabolism of the joint is the articular chondrocyte, which undergoes a series of complex changes, including hypertrophy, proliferation, catabolic alterations and ultimately, death. Many of these changes are induced by cytokines and reactive oxidant species produced by the chondrocytes themselves, which are key protagonists in the autodestruction of articular cartilage (Pelletier *et al*., 2001; Aigner *et al*., 2002).

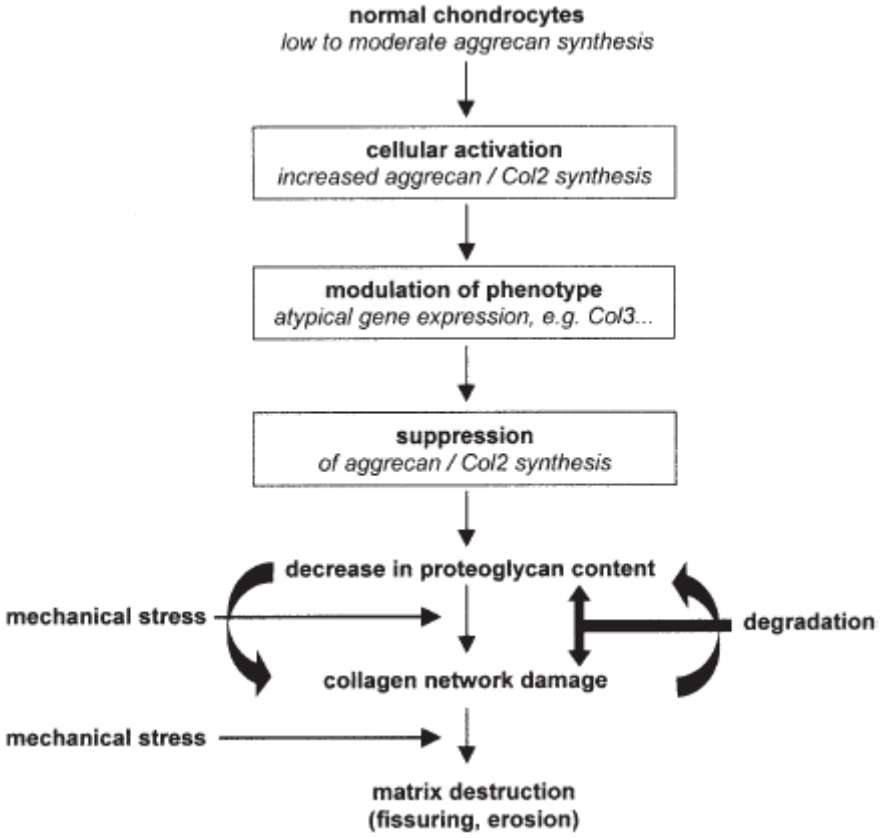

Fig. 4: Schematic representation of three steps in a hypothetical pathomechanism involved in the progression of osteoarthritic cartilage degeneration: (i) cellular activation of chondrocytes, (ii) modulation of the cellular phenotype, and (iii) suppression of anabolic activity. The latter leads to a quantitative loss of aggrecan molecules from the ECM and – via mechanical stress – to collagen network damage, which again promotes further loss of proteoglycans. Finally, fissuring and complete destruction of the cartilage matrix occurs. Figure reproduced from Aigner and McKenna (2002).

Inflammatory mediators including cytokines, prostanoids and NO are generally agreed to induce cartilage degradation (Häuselmann, 1997; Arend, 2001). The inflammation in that case primarily takes place in the synovial membrane and the destruction of cartilage is a secondary event. In non-inflammatory arthropathies, such as OA, synovial cell reactions toward components released from cartilage into the synovial fluid may contribute to disease progression (Pelletier *et al*., 1995; Smith *et al*., 1997), but the major pathogenic processes are localized within the cartilage itself (Amin *et al*., 1999; Moos *et al*., 1999). Chondrocytes in OA affected cartilage display enhanced and coordinated expression of pro-inflammatory cytokines and inducible NO synthase iNOS, the enzyme responsible for NO production (Melchiorri *et al*., 1998). Collectively, evidence is accumulating that mediators of inflammation acting in an autocrine/paracrine fashion within the cartilage play a primary role in the pathogenesis of OA (Towle *et al*., 1997; Melchiorri *et al*., 1998; Amin *et al*., 1999; Moos *et al*., 1999).

It has also been proposed that the inorganic free radical NO is a potential factor in the promotion of cartilage catabolism in OA. Compared with the normal state, OA cartilage produces a large amount of NO, both under spontaneous and pro-inflammatory cytokine-stimulated conditions (Pelletier *et al*., 1996). A high level of nitrite/nitrate has been found in the synovial fluid and serum of arthritis patients (Farrell *et al*., 1992; McInnes *et al*., 1996). This is caused by an increased level of the inducible form of NO iNOS, the enzyme responsible for NO production (McInnes *et al*., 1996; Grabowski *et al*., 1997). As such, an increased level of IL-1β, in conjunction with a decreased IL-1ra-level, may cause an over-stimulation of OA chondrocytes by this factor, leading to enhanced cartilage matrix degradation. Interestingly, a selective inhibitor of iNOS administered *in vivo* proved to exert positive therapeutic effects on the progression of lesions in an experimental canine OA model (Pelletier *et al*., 1998).

1.10. Therapeutic measures in OA

OA is chronic, incurable, and expensive disease lacking effective treatment. A complete therapy for OA is still elusive and long-term usage of non-steroidal anti-inflammatory drugs or NSAIDs (Bannwarth, 2006) to alleviate pain is commonly associated with gastro-intestinal tract disturbances and drug intolerance. Moreover, NSAIDs may not be a good choice in chronic conditions and are substituted by COX-2 inhibitors. Systemic, long-term intake of these COX-2 inhibitors has raised questions on side effects in the system, as these are proven to block the entire synthesis of prostaglandins. Recent improvements in the knowledge of the pathophysiology of OA have led to the development of biological treatments. Marketed agents for biological therapy fall into two categories: TNFα and IL-1β inhibitors. These biological treatments provide significant efficacy in the short and medium term treatments in many patients. However, the molecules are complex and expensive to manufacture.

As an alternative and also to circumvent these problems with NSAIDs and COX-2 inhibitors, gene therapy is fast emerging to become a potential force. The concept is based on over-expression of anti-inflammatory genes in chondrocytes, which are represented by Th_2 cells. Previous studies have shown that IL-4 has more advantages over IL-10 in down-regulating the inflammatory cascade in OA and hence has been chosen as one of the candidate genes (Relic *et al*., 2001). This

concept aims at the re-establishment of homeostasis in the knee joint by restoring the balance between Th_1 and Th_2 cells.

Although available biological treatments for OA are effective, their inherent risks as well as high costs are major limitations. An attractive alternative lies in gene therapy. Overall, published studies have firmly established the scientific validity of gene therapy in OA models. Nevertheless, advances and refinements are needed to define the reference strategy. To this end, further experimental and preclinical studies must be conducted. Difficulty arises in selecting whether viral or non-viral vectors should be used for the transfer of the therapeutic candidate gene into the system. While viral vectors provide higher transfection efficiency, the main drawback has been the immunogenicity of viral gene products. On the other hand, the non-viral methodology is safer but less efficient. With the advent of nucleofector technology from Amaxa, this drawback of the non-viral method has been greatly reduced. Usually non-viral vector methods are termed as *ex vivo*, as the cells are first extracted, then transfected with genetic material, and finally implanted back into the system. On the other hand, viral vectors could be given directly at the site of therapy by means of an injection and this is usually termed as *in vivo* in gene therapy. Although the *ex vivo* method is cumbersome, no complications have been yet reported and thus it is mostly preferred over the use of viral vectors.

1.10.1. Potential therapeutic applications of cytokine modulation in OA

The neutralization of IL-1 and/or TNFα up-regulation of MMPs gene expression appear to be a logical development in the potential medical therapy of OA. Indeed, recombinant IL-1ra and IL-1sr proteins have been tested in both animal models of OA for modification of OA progression. Soluble IL-1ra suppressed MMP-3 transcription in the rabbit synovial cell line HIG-82. Experimental evidence showing that neutralizing TNFα-suppressed cartilage degradation in arthritis also supports such strategy. The important role of TNFα in OA may emerge from the fact that human articular chondrocytes from OA cartilage expressed a significantly higher number of the p55 TNFα receptor that could make OA cartilage particularly susceptible to TNFα degradative stimuli. In addition, OA cartilage produces more TNFα and TNFα convertase enzyme (TACE) mRNA than normal cartilage. By analogy, an inhibitor to the p55 TNFα receptor may also provide a mechanism for abolishing TNFα-induced degradation of cartilage ECM by MMPs. Since TACE is the regulator of TNFα activity, limiting the activity of TACE might also prove efficacious in

OA. IL-1 and TNFα inhibition of chondrocyte compensatory biosynthesis pathways which further compromise cartilage repair must also be dealt with, perhaps by employing stimulatory agents such as transforming growth factor β (TGFβ) or insulin like growth factor-1 (IGF-1). The capacity of IL-1ra to reduce *in vitro* and *in vivo* cartilage degradation, MMP production and the progression of OA lesions (Caron *et al*., 1996; Pelletier *et al*., 1997) has elicited much attention concerning the use of this molecule in OA therapy, and more particularly in regard to gene therapy. The IL-1ra gene has been successfully transferred into animal and human synovial cells using an *ex vivo* technique (Evans and Robbins, 1994; Baragi *et al*., 1995). One such study using the experimental dog model of OA showed *in vivo* that the progression of structural changes of OA was significantly reduced (Pelletier *et al*., 1997). It has also been demonstrated *in vitro* that the human IL-1ra gene can be successfully transferred into chondrocytes, and that the resulting increase in production of IL-1ra can protect the OA cartilage explants from degradation induced by IL-1 (Baragi *et al*., 1995).

The knowledge of the inflammatory cascade in OA serves as a good cause for choosing gene therapy. The other reason being that OA is not life threatening and that the inflammation is restricted to the knee joint. Further, OA has not been shown to lead to secondary infections, implying that cytokines produced within the knee joint are not distributed systemically. Another important aspect has been the avascular and aneural nature of cartilage (Upholt and Olsen, 1991). Due to this fact, systematic complications may be avoided by gene therapy limited to cartilage. Moreover, chondrocytes are the only cells present in cartilage and are easily extracted and propagated in cell culture. *In vitro* propagation and growth on scaffolds make these cells ideal for being replanted into the knee joint. These aspects clearly put OA in a different class to those diseases which have shown serious repercussions as a result of gene therapy. What remains elusive is the regulated expression of the therapeutic candidate gene. Typically, gene therapy should simulate the effects of systemic intake of medicines, but at the same time should be confined to the site of inflammation. In other words, the therapeutic candidate gene should be expressed at the site of OA occurrence and only in the presence of inflammation and expression should ideally be regulatory.

A novel and interesting approach to controlling pro-inflammatory cytokine production and/or activity is the use of biological molecules possessing anti-inflammatory

properties. Augmenting inhibitor production *in situ* by gene therapy or supplementing it by injecting the recombinant protein is an attractive therapeutic target, although an *in vivo* assay in OA is not available, and its applicability has yet to be proven. As such, recombinant human IL-4 has been tested *in vitro* on OA synovial tissue, and has been shown to suppress the synthesis of both IL-1β and TNFα in the same manner as low dose dexamethasone (Bendrups *et al*., 1993). To date, of the anti-inflammatory cytokines, only IL-10 is employed in clinical trials for the treatment of RA in humans. Results from IL-13 experimentation on human synovial membrane from OA patients (Jovanovic *et al*., 1998) indicate it is potentially useful in the treatment of this disease.

The understanding of the release and activity of cytokines has evolved greatly in recent years, and a clearer comprehension of their modulation of factors as well as their major regulations has helped to more accurately identify effective targets that may be potentially therapeutic in the treatment of OA. This may occur via a direct or indirect decrease in the release and/or action of these factors, and various cytokine-related therapies are now being considered. Novel approaches include the inhibition of IL-1β and TNFα production. Chemical and biological agents are currently being evaluated. These include inhibitors of the enzyme responsible for the conversion of IL-1β to an active molecule (ICE), soluble cytokine receptors (IL-1ra, anti-inflammatory cytokines (IL-4, IL-10, IL-13); and targeting the intracellular signaling cascades or transcription factors of IL-1β or TNFα (Martel-Pelletier, 1999).

1.10.2. Gene therapy

Developing gene-based therapy for OA in both human and veterinary patients represents an exciting challenge. The detailed understanding of disease pathogenesis has already allowed the introduction of ‘’structure modifying’’ therapeutic genes into arthritic joints to disease progression at the molecular level by inhibiting the enzymes responsible for cartilage degradation while enhancing tissue repair (Goldring and Goldring, 2007).

However, despite considerable advances in molecular biology, several technical hurdles must be overcome before gene therapies can be considered acceptable clinical practice. In the development of a gene-based therapy for OA, appropriate therapeutic genes and vector vehicles must be selected and methods devised for efficient delivery and sustained expression. However, it is also necessary to minimize undesirable side-effects by accurately targeting therapeutic gene expression to

diseased cells. To this end it is possible that regulatory promoter elements can be modulated to enhance therapeutic levels of gene expression whilst maintaining both disease and cell type specificity.

The construction of vectors enabling tissue-specific gene expression is one of the current challenges in the field of gene therapy. A number of transcriptionally based targeting strategies have been described which target specific cell types or are targeted through specific pathologies within the cell. However, correctly regulated expression may not only require promoter regions but also the distant 5' and 3' elements that influence tissue-specific promoter activity (Miller and Vile, 1995). Tissue-specific regulatory elements have already been used to target gene expression to certain cell types. For example, using the transgenic mouse model for muscular dystrophy, the creatine kinase promoter has been used to restrict dystrophin cDNA expression to skeletal and cardiac muscles to correct the clinical signs of disease without deleterious side-effects (Cox *et al*., 1993). However, vector context is an important parameter when designing tissue-specific targeting systems. Although tissue-specific promoters frequently retain their specificity in the context of retroviral vectors (Hatzoglou *et al.*, 1990) this is not always the case, and the design of the viral vectors may have significant effects on cell type specificity due to promoter interference (Vile *et al*., 1994).

1.10.3. Transplantation and transduction of chondrocytes

The greatest obstacle to develop efficient gene-transfer protocols targeting sites of articular cartilage damage to date has seen the restrained accessibility of the defects and the gene vehicles capable of transducing chondrocytes within their native matrix. The past decade has been significant progress in a number of areas to address these issues. Experimental approaches currently employed to transfer genes to articular cartilage defects are shown in Figure 5.

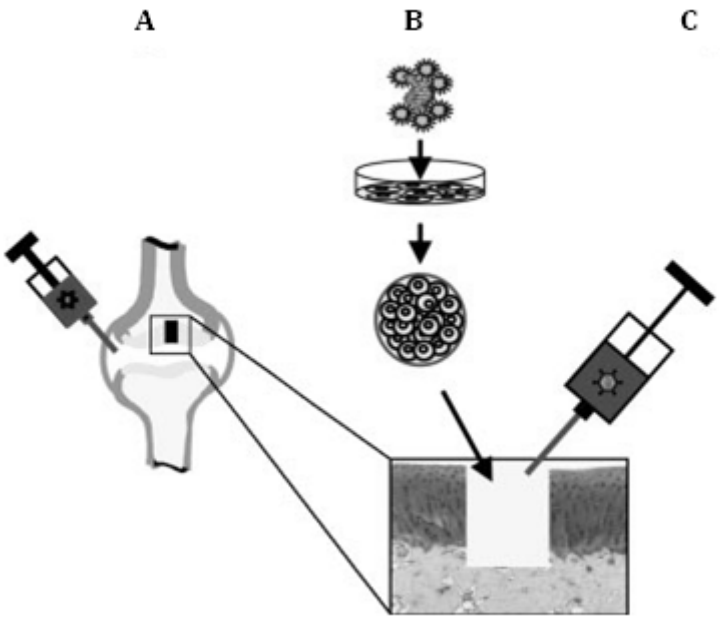

Fig. 5: Strategies to transfer genes into articular cartilage defects: These strategies include (A) the injection of a gene vehicle or of genetically modified cells into the joint space; (B) arthrotomy (open joint surgery) and transplantation of *ex vivo* genetically modified cells into the articular cartilage defect (*ex vivo* approach); or (C) arthrotomy with direct administration of gene vectors into the articular cartilage defect (*in vivo* approach). With intra-articular injection, it is not feasible to deliver a gene shuttle vector or genetically modified cells specifically into a focal cartilage defect. When using the *ex vivo* approach, the modified cells are usually implanted in conjunction with supportive matrices in order to contain them in the defect (e.g. as an alginate sphere). Direct administration of gene vectors to a cartilage defect mainly results in transduction of the cells that spontaneously fill the defect. Figure with legends reproduced from Cucchiarini and Madry (2005).

The direct transplantation of cells genetically modified *ex vivo* into an isolated articular cartilage defect is a step to deliver therapeutic genes more precisely within the cartilage lesions. This procedure involves the isolation of autologous or allogenic articular chondrocytes or other cell types (e.g. bone marrow derived mesenchymal cells, fibroblasts), followed by the modification of these cells *ex vivo* and their re-implantation.

The use of alginate as a three dimensional carrier system has the advantage to be already applied in human studies (Soon-Shiong *et al*., 1994). The alginate system (Bonaventure *et al*., 1994; Lemare *et al*., 1998; Madry *et al*., 2003, Cucchiarini and Madry, 2005) was preferred over other three dimensional culture systems also due to its well-characterized ability to maintain the differentiated phenotype of chondrocytes (von der Mark *et al*., 1977; Benya *et al*., 1978), the comparability of its negative charge density to that of native cartilage matrix, and its stability over time

(Bonaventure *et al*., 1994). Based on these facts encouraging results have been obtained using alginate composites for transgene expression in articular cartilage defects by various researchers (Diduch *et al*., 2000; Fragonas *et al*., 2000; Madry *et al*., 2003; Madry *et al*., 2005; Kaul *et al*., 2006). In most cases, the knee joint was entered through a medial para-patellar approach. The patella was dislocated laterally and the knee flexed to 90^{o} and cylindrical osteochondral defects were created in each patellar groove with a manual cannulated burr. Alginate-chondrocyte spheres transfected with expression vectors were press-fit into the defects. Using alginate spheres as a carrier systems yielded very useful results (Cucchiarini and Madry, 2005).

1.10.4. Chondrocyte differentiation and modulation

A number of current therapeutic protocols for correction of focal cartilage defects and possible future treatment of OA involve the expansion of autologous chondrocytes followed by re-implantation of these cells into cartilage defects, their injection into affected joints, or their utilization for engineering of replacement tissue *ex vivo* (Brittberg *et al*., 1994; Buckwalter and Mankin, 1998). All of these protocols require an expansion phase of chondrocytes in culture in order to obtain sufficient cells to implement treatment. However, it is well known that primary mammalian cells in culture have a finite replicative life span and eventually enter a state of senescence in which they remain metabolically active but cease to proliferate. Furthermore, the mitotic potential of primary cells in culture is dependent on the age of the donor, with cells from older individuals exhibiting a lower proliferative life span (Hayflick, 1965). Normal adult chondrocytes also possess a limited mitotic potential and inevitably enter a state of replicative senescence in which cellular proliferation ceases (Adolphe *et al*., 1983; Evans and Georgescu, 1983; Martin and Buckwalter, 2001).

It has been demonstrated that chondrocytes from various species show a relationship between the number of population doublings achieved *in vitro* and the life span of the organism (Adolphe *et al*., 1983). Recent work has shown that both the proliferative activity and the telomere length of articular chondrocytes decrease with the age of the donor (Martin and Buckwalter, 2001). Indeed, chondrocytes from articular cartilage exhibit a number of age-related changes in their phenotype. Among these changes are decreased response to growth factors such as TGFβ and IGF-1, increased apoptosis, and decreased ECM production (Guerne *et al*, 1995; Adams and Horton, 1998). Compounding the latter events are the phenotypic changes in

chondrocytes that occur during OA, a disease that has a high correlation with age (Abyad and Boyer, 1992; Hamerman, 1993). These alterations, collectively, place a limit on the usefulness of autologous chondrocytes isolated from aged OA joints in the therapeutic strategies mentioned above.

After enzymatic isolation of the chondrocytes from cartilage tissue, synthesis of collagen and proteoglycan is up-regulated. When cultured in monolayer on tissue culture plastic, the rapidly dividing round chondrocytes lose their cartilage phenotype and transform into flattened fibroblast-like cells, and lack their morphological and biochemical characteristics (Benya *et al*., 1977; Benya *et al*., 1978). In fact, the modulated chondrocytes gradually start to synthesize type I and III collagen instead of type II collagen (Benya and Shaffer, 1982; Bonaventure *et al*., 1994; Schnabel *et al*., 2002). There is also a gradual change from the synthesis of large aggregating proteoglycans (aggrecan) to low molecular weight proteoglycans (von der Mark, 1986). This shift in cellular differentiation has been demonstrated both by morphological changes and by alterations in collagen expression patterns (Fuss *et al*., 2000; Stewart *et al*., 2000). The de-differentiation process is believed to be mediated by the formation of actin stress fibers which occurs when the cells spread on an adhesion-permitting substrate (Mayne *et al*., 1976; Benya *et al*., 1978).

1.10.5. Three dimensional (3D) culture systems

Among the various directions explored in order to have a large number of differentiated articular chondrocytes easily available, the restoration of the differentiated properties after cell multiplication in monolayer has been proposed. Chondrocytes in 3D culture produce ECM with morphologic and functional characteristics similar to those of articular cartilage (Thompson *et al*., 1985; Buschmann *et al*., 1992; Cook *et al*., 1997). The morphological changes caused by suspension culture are accompanied by simultaneous down-regulation of genes that are characteristic of proliferating fibroblast and an up-regulation of cartilage specific genes, a process termed re-differentiation, which occurs over a period of several weeks (Yaeger *et al*., 1997; Binette *et al*., 1998). The restoration of the expression of some phenotypic markers of the articular chondrocyte has been shown by culturing de-differentiated cells at high density (Borge *et al*., 1996), in agarose (Benya and Shaffer, 1982) and collagen (Thenet *et al*., 1992) gels, in alginate microspheres (Bonaventure *et al*., 1994) or in the presence of dihydrocytochalasin B (Benya *et al*., 1988) or TGFβ (Benya and Padilla, 1993). Such culture systems maintain the

chondrocytes in a rounded morphology and prevent the formation of stress fibers (Takigawa *et al*., 1984; Benya *et al*., 1988; Brown and Benya, 1988; Newman and Watt, 1988; Loty *et al*., 1995).

It has also been found that the calcium alginate suspension cell culture system is useful for studying the influence of different factors on chondrocyte properties in general and the synthesis and turnover of proteoglycans in particular (Häuselmann *et al*., 1992). During alginate culture the chondrocytes divide to form cell clusters and also synthesize cartilage-specific matrix components which form a halo around the cells (Almqvist *et al*., 2001; Kikuchi *et al*., 2001). While cell numbers increase during initial culture, a plateau is reached at a later time indicating an inhibition of cell proliferation, which may involve processes similar to contact inhibition in monolayer cultures (Guo *et al*., 1989; Beekman *et al*., 1997; Enobakhare *et al*., 2001). Individual cells can be recovered by dissolving the alginate, using calcium chelating agents followed by enzymatic digestion to remove elaborated matrix, allowing repeat passage (P) to be performed (Madry *et al*., 2003). Chondrocytes grown in alginate regain their characteristic spherical shape and re-express chondrocyte specific proteins (Guo *et al*., 1989; Lemare *et al*., 1998; Liu *et al*., 1998). Related more to the *in vivo* situation, culture of chondrocytes in alginate beads appears particularly promising, because the modulation can be prevented for several months and the cell-associated matrix formed de novo is apparently very similar to native cartilage matrix in its composition (Häuselmann *et al*., 1996; Petit *et al*., 1996).

Culturing chondrocytes in alginate beads resulted in the re-expression of the two main markers of differentiated chondrocytes: aggrecan and type II collagen gene expression was strongly re-induced from day 4 after alginate inclusion and paralleled protein expression. However, 2 weeks were necessary for total suppression of type I and III collagen synthesis, indicators of a modulated phenotype. IL-1β, a cytokine that is present in the synovial fluid of RA patients, induces many metabolic changes on the chondrocyte biology. Compared with cells in primary culture, the production of NO in response to IL-1β was impaired in cells at P2 in monolayer but was fully recovered after their culture in alginate beads for 2 weeks. This suggested that the effects of IL-1-1β on cartilage depend on the differentiation state of chondrocytes. This makes the culture in alginate beads a relevant model for the study of chondrocyte biology in the presence of IL-1β and other mediators of cartilage destruction in RA and OA (Lemare *et al*., 1998).

The alginate system (Bonaventure *et al*., 1994; Lemare *et al*., 1998; Madry *et al*., 2003, Cucchiarini and Madry, 2005) was preferred over other 3D culture systems due to its well-characterized ability to maintain the differentiated phenotype of chondrocytes that is lost after passaged long-term monolayer culture (von der Mark *et al*., 1977; Benya *et al*., 1978), the comparability of its negative charge density to that of native cartilage matrix, and its stability over time (Bonaventure *et al*., 1994). The alginate culture system for chondrocytes is a convenient model for studying synthesis and extracellular deposition of ECM (Beekman *et al*., 1997).

1.10.6. Immortalization of primary chondrocytes

Numerous studies have shown that shortening of telomere length, which occurs during each cell division, is probably a signal for cellular senescence, since cells in which telomere length has been shortened to a critical level fail to undergo further mitotic events (Greider, 1990; Harley *et al*., 1990; Allsopp *et al*., 1992). The enzyme telomerase is a reverse transcriptase that solves the problem of DNA end-replication, and therefore affects telomere shortening due to the inability of eukaryotic DNA polymerases to completely replicate the ends of linear chromosomes (Bryan and Cech, 1999; Meyerson, 2000). Human telomerase is a ribonucleoprotein consisting of a reverse transcriptase protein subunit and an RNA template subunit (Feng *et al*., 1995; Lingner *et al*., 1997). This RNA subunit encodes ~1.5 telomeric repeats, and thereby acts as a template for the human telomerase reverse transcriptase (hTERT) to add new telomeric sequences onto the chromosome ends. However, most human somatic cells no longer express telomerase and it is believed that the absence of this activity is a major contributing factor to their finite replicative life span (Kim *et al*., 1994; Shay and Bacchetti, 1997).

The life span of certain human somatic cell types (skin fibroblasts, endothelial cells, and retinal pigment epithelial cells) has been extended in culture by the ectopic expression of the hTERT protein (Bodnar *et al*., 1998; Jiang *et al*., 1999; Morales *et al*., 1999; Yang *et al*., 1999; Steinert *et al*., 2000). The immortalized cells maintained their differentiated phenotype and did not acquire transformed or malignant characteristics (Jiang *et al*., 1999; Morales *et al*., 1999). Interestingly, the extension of the life span of skin fibroblasts and retinal pigment epithelial cells by introduction of telomerase did not require any of the other components of the telomere-lengthening machinery such as telomere binding proteins or the RNA template component (Bodnar *et al*., 1998; Yang *et al*., 1999; Steinert *et al*., 2000). These results imply that

the life span of a wide variety of somatic cells can be extended simply by the ectopic expression of telomerase, and support the telomere hypothesis of cellular aging which proposes that most somatic cells become senescent because of progressive shortening of their telomeres with each cell division.

In addition to the problem of phenotypic drift, studies are often limited, because cartilage has low cellularity and is present in low quantity throughout the organism. The availability of normal chondrocytes is even more critical when they are of human origin. So, various directions have been explored in order to make available large number if differentiated articular chondrocytes. Immortalization has been tried, but stable retention of a differentiated chondrocytic phenotype has been elusive (Thenet *et al*., 1992; Mallein-Gerin and Olsen, 1993; Goldring *et al*., 1994).

1.11. Canine OA as a potential animal model

The full spectrum of mechanical, morphologic, and metabolic changes that occur in articular cartilage throughout the course of OA is difficult to appreciate based on studies of human pathology, which typically focus on the end stages of this disease. Experimental animal models of OA provide joint tissues for study throughout the natural history of a disease process that mimics, to some extent, human and veterinary OA. OA is a chronically progressive articular disease that is characterized by both degeneration (destruction) of cartilage and proliferative (or regenerative) changes in bone and cartilage. Human studies are of limited use in investigating the pathology of this disease, and for this reason research has been conducted using experimental animal models (Miyashita *et al*., 2000). These models all have potential use in the study of molecular mechanisms associated with OA development and apply immunohistochemistry, biochemistry and molecular probes to identify altered matrix molecules at different stages in disease progression (Bendele, 2002). Most of the information in the literature on changes, which occur in articular cartilage in the early stages of OA, has been obtained from animal models (Adams and Billingham, 1982).Such studies have been used with the aim to gain insight into the pathogenesis of OA, and the major conclusions so far have been that the cartilage destructive process is mainly IL-1β driven, whereas TNFα is involved in the onset of arthritis (Van den Berg, 1997; Goldring, 1999).

The validity of dog model has been tested by *in vitro* studies whereby canine chondrocytes have been the area of great interests. Cook *et al*. (2000) and Kuroki *et al*. (2004) have used IL-1β and TNFα to 3D cultured canine chondrocytes or cartilage

explants and concluded that they provide an appropriate *in vitro* model for at least a subset of the pathophysiological events associated with OA. In addition, studies that use such models could lead to further elucidation of distinctive roles of the pro-inflammatory cytokines in the OA disease process. Therefore, use of such *in vitro* model based on canine chondrocytes has been aimed in this study.

1.12. Rationale and Aims

A great deal of research has been focused on understanding the mechanisms that induce metabolic changes in articular chondrocytes during various pathologies such as in OA and RA. For this purpose, articular chondrocytes in culture are a very useful tool. However, a major drawback is that the cellularity of cartilage is low and subculture in monolayer or two-dimension (2D) culture system, in order to increase the cell number, is not possible.

Pro-inflammatory cytokines are believed to play a pivotal role in the initiation and development of this disease process, among which IL-1β and TNFα appear prominent. IL-1β is extremely important to cartilage destruction, while TNFα appears to drive the inflammatory process (Van de Loo *et al*., 1995; Caron *et al*., 1996).

Taken in aggregate, published studies have firmly established the scientific validity of gene therapy in OA/RA models. Although available biological treatments are effective, their high cost is a major limitation. Alternatives must be found in the fields of chemotherapy or biological therapy; gene therapy may be among these alternatives. It appears that viral strategies using new vectors such as adeno-associated virus are highly efficient and have many theoretical advantages for a clinical development. Non-viral strategies, in addition, have a fascinating preclinical development in arthritis. Clinical trials are introduced into research programs as soon as a treatment strategy shows a reasonable likelihood of being at least as effective as a reference treatment. Occurrence of a serious adverse event could cause irreparable harm to scientific and clinical research on gene therapy by generating uncontrollable reactions among the general public and research investors. These considerations and the large amount of preclinical work needed explain why few clinical trials are under way (Boissier, 2001). Clearly, gene therapy is applicable to OA/RA, although much more work is needed to prove that gene therapy can improve functional impairment and quality-of-life in patients with this disease.

Development of a new strategy for treatment of OA in small animal medicine, which could be forerunner of similar treatments in human OA, is warranted and contribution

between IL-4 anti-inflammation treatments with regulatory aspects (disease controlled) is required.

1.12.1. Molecular and phenotypic modulation of articular chondrocytes

Canine articular chondrocytes will be isolated from cartilage tissues and will be characterize in order to evaluate the progressive changes that they undergo in serial monolayer cultures. The time course of de-differentiation and the potential for re-differentiation after transfer into alginate matrix will be investigated, the object being to define an optimal time for harvesting cells for further applications such as transfection efficiency and cytokine studies. Using biochemical methods and recombinant DNA-technology, evidence will be presented of how the chondrocyte phenotype, the growth behaviour and the expression of selected cartilage-specific molecules are modulated by 2D and alginate-based 3D culture systems. Given the importance of extending the proliferative capacity and delaying the occurring of senescence in articular chondrocytes, efforts will be made to create an immortalized chondrocyte cell line using the human telomerase catalytic subunit.

1.12.2. Development and characterization of a 3D model of inflammatory arthritis

IL-1β and TNFα, the two master pro-inflammatory cytokines involved in OA pathophysiology, will be cloned in a bacterial system and respective canine recombinant proteins will be purified in order to use them as species specific stimulants in canine chondrocytes. An *in vitro* model of OA will be established by use of canine chondrocytes in 3D culture with canine recombinant IL-1β and TNFα. This model will be established in order to provide a subset of the pathophysiologic events associated with naturally occurring OA and to allow analysis of the effects of these cytokines on the biochemical processes associated with OA.

1.12.3. Expression and characterization of IL-4 transgene in chondrocytes

The available canine IL-4 gene will be transduced in canine chondrocyte to monitor protein expression in this mammalian system. An ELISA (enzyme linked immunosorbent assay) system will be setup to quantify the expressed protein. The biological activity of IL-4 will be assessed by its capacity to suppress a series of selected inflammatory mediators in a 3D model of inflammation where chondrocytes will be transfected with IL-4 and stimulated by exogenous addition of IL-1β and TNFα.

2.0. MATERIALS AND METHODS

2.1. Molecular and phenotypic characterization of chondrocytes

2.1.1. Cartilage tissue collection

Articular cartilage tissues were collected 24 h post-euthanasia from macroscopically normal knee joints of canine cadavers (n = 5) with no history of joint disease. The joint cartilage was kept intact under cover of muscles/tissues and the samples were prepared for cell isolation under a laminar flow hood.

2.1.2. Isolation of chondrocytes

Chondrocytes were isolated following the protocol previously described with slight modifications (Kaps *et al.,* 2004). Briefly, the fascia and muscles covering the joint were removed, joint was cleaned with 70% ethanol and cartilage was sliced by careful dissection. Precautions were exercised to avoid inclusion of the subchondral bone in cartilage pieces. Samples of cartilage were collected in Dulbecco's Modified Eagle Medium (DMEM) enriched with 10% heat inactivated fetal calf serum (FCS), 100 U ml^{-1} penicillin and 100 mg ml^{-1} streptomycin. Cartilage pieces after weighing were washed three times with HANKS' salt solution, once with 70% ethanol for 15 sec and twice with DMEM with 10% FCS and 1% penicillin/streptomycin. For slicing the samples were placed in a Petri dish containing fresh medium. Two sterile scalpels were used to dice the cartilage into 1-3 mm^3, pieces. The diced tissue was placed in growth medium with sterile filtered collagenase P (1 U ml^{-1}), collagenase CLS II (330 U ml^{-1}) and hyaluronidase (30 U ml^{-1}) in a spinner flask. The flask was placed on an orbital shaker and incubated at 37^{o}C with 5% CO_2. Approximately 16-18 h post-incubation, the digestate was resuspended, filtered through nylon mesh (pore size 100 µm) into a sterile tube and centrifuged at 400xg for 20 min to pellet the cells i.e. chondrocytes. The cell pellet was gently washed three times in HANK'S salt solution and finally resuspended in growth medium. Chondrocytes were counted and their viability assessed using a Neubauer chamber and trypan blue exclusion staining based on four counts per sample.

2.1.3. Primary culture in monolayer

Isolated chondrocytes were seeded in tissue culture plates or flaks as the primary culture (primo) at a density of $2x10^4$ cells/cm^2. The growth medium for all cultures was DMEM with 10% FCS and 1% penicillin and streptomycin unless otherwise indicated. The growth medium was changed 2-3 times per week after washing the

cells twice with 1x calcium-free and magnesium-free PBS solution. Cells were cultivated at 80% confluence and passaged seven times by the release from the dishes with a solution of 0.1% trypsin and 0.1% EDTA at 1:3 ratios. All cultures were grown in a humidified CO_2 incubator under 95% air and 5% CO_2 at 37^{o}.

2.1.4. Cryopreservation of chondrocytes

Freshly isolated and passaged chondrocytes were cryopreserved at each passage to stock them for future use. For this purpose, approximately $1x10^6$ cells ml^{-1} were suspended in growth medium (without antibiotics) containing 10% (v/v) highest purity dimethylsulfoxide (DMSO). The cell suspension was put into cryopreservation ampoules placed in an isopropanol bath and subsequently stored at -80°C. After 2-3 weeks the ampoules were transferred to boxes merged in liquid nitrogen (-196^{o}C) container until further use.

2.1.5. Three-dimensional (3D) culture

Cryopreserved chondrocytes were thawed in a water bath at 37^{o}C for 1 min, seeded in tissue culture flasks and medium was changed immediately after the cells settled at the bottom of flask. Cultivation was continued until they reach approximately 80% confluence. Cells were trypsinized, washed and centrifuged at 200xg for 5 min to pellet them. For encapsulation of chondrocytes in alginate microspheres previously described protocol was followed (Madry *et al.,* 2003). Briefly, chondrocytes were suspended in sterile filtered 1.2% alginate in 0.15 M NaCl at $5x10^5$ cells ml^{-1}. The cell suspension was then extruded through a 21-gauge needle into a 102 mM $CaCl_2$ solution at room temperature (RT) under constant shaking and the chondrocytes-alginate composite was allowed to polymerize for 10 min. The resulting implants were then washed twice in 0.15 M NaCl followed by two consecutive washes in basal medium and placed in growth medium. Alginate microspheres were incubated in growth medium which was changed three times per week. Entrapped chondrocytes were released from alginate beads after each week for three weeks. Individual microspheres were solubilized by incubation in 100 µl 55 mM sodium citrate, 90 mM NaCl (pH 6.8) for 20 min at RT. The released chondrocytes were counted and their viability assessed as before.

2.1.6. Growth characteristics of chondrocytes

Chondrocytes cultured in 2D in P0-P7 and were monitored for growth characteristics using a number of parameters. These includes growth rate, cell size and granularity

profiles and characteristics chondrocyte morphology. The cells were monitored on a daily basis to record their phenotype and were photographed at each passage. The growth rate during cultivation was determined using the growth constant k = (InN_2 – InN_1)/t (Fröhlich *et al.,* 2007) in which InN_1 was the initial number of cells ($1x10^6$/25 cm^2), InN_2 was the number of cells in confluent cultures and *t* was the number of days cells reached confluence. For growth rate $1x10^6$ in P0 were cultured in growth medium and incubated under standard incubation conditions. When chondrocytes attained a confluence, were trypsinized and counted to record average cell number. Similarly, $1x10^6$ cells in P5 were cultured on same lines for the next passage and experiment was continued until P7. Average cell number and growth constant *k* were calculated for each passage.

2.1.7. Phenotype of chondrocyte

Chondrocyte morphology was first analyzed visually by light microscopy of confluent cultures. Flow cytometry analyses of chondrocytes were done to estimate the cell size and granularity on long term culture using fluorescence activated cell sorting (FACS). Chondrocytes were harvested after trypsinization and a total of $3x10^5$ cells were suspended in 1 ml of FACS flow sheath fluid. For viability test 1 µl of propidium iodide exclusion dye was added in the cell suspension. The morphological profile of the cells was observed by combining forward light scatter (FS) and orthogonal or side light scatter (SS). The FS measurement was related to the cell size while SS was associated with internal granularity (Fröhlich *et al.,* 2007).

2.1.8. Histological and immunohistochemical analysis of cartilage tissue

Cryostat sections (3-5 µm thick) were cut from cartilage tissue and frozen at –70°C. Sections, on super-frost slides were fixed for 5 min in ice-cold acetone and then stored at –20°C until required for alcian blue staining which reacts with proteoglycans, a component of cartilage matrix, or for immunostaining to detect collagen in the tissue.

The cryosections from the central part of the tissue were mounted on aminoalkylsilane-coated slides. Cartilage matrix proteoglycans were stained with alcian blue 8GX at pH 2.5 and counterstained with nuclear fast red according to the method of Romeis (1989) as previously described (Lübke *et al.,* 2005).

For the detection of type I collagen or type II collagen, cryosections were incubated with goat anti-collagen type I polyclonal (dilution, 1:1000) or rabbit anti-bovine type II

collagen polyclonal antibody (dilution 1:200). The slides were then washed three times with PBS^- and incubated for 30 min at 37°C with LSAB/LINK, washed again two times and treated for 30 min with LSAB/HRP streptavidin. Specimens were then developed with diaminobenzidine (liquid DAB) and counterstained with haematoxylin (Kaps *et al.,* 2004).

2.1.9. Immunohistochemical analysis for collagens

The immunocytochemical analyses for the expression of type I collagen and type II collagen in chondrocytes was performed using the Dakocytomation system as described previously (Kaps *et al.,* 2004) with slight modifications. Briefly, $1x10^5$ chondrocytes at P1-P7 were cultivated on eight-chamber slides with growth medium until they were 60-70% confluent. The cultivated cells were washed thrice with PBS, fixed with acetone/methanol (1:1) for 5 min and were set to air dry for 30 min. Then H_2O_2 was added for 5 min and subsequently washed three times with PBS. Slides were incubated overnight at 37°C with goat anti-collagen type I (dilution, 1:1000) or rabbit anti-bovine type II collagen (dilution 1:200) antibodies diluted in antibody diluent with background reducing components. Afterwards, the slides were washed thrice with PBS, incubated for 30 min at 37°C with LSAB/LINK, washed twice in PBS and treated for 30 min with LSAB/HRP streptavidin. Specimens were developed with diaminobenzidine (DAB) and counterstained with hematoxylin. Slides were photographed and subjectively evaluated in duplicate by two investigators.

2.1.10. RNA isolation and cDNA synthesis

A total of $5x10^5$ cells from each passage in 2D and cells released from alginate microspheres at each week were centrifuged at 200xg for 10 min. Cell pellets were washed with PBS and kept at –80°C until used for total RNA isolation. Total RNA was isolated using TRIsure reagent (Bioline, Germany) according to supplied protocol. Then 1 µg of total RNA was subjected to DNase degradation to eliminate residual genomic DNA and was reverse transcribed to synthesize first strand cDNA (Invitrogen, Germany) according to manufacturer's instructions. The concentration and purity of samples were determined using Nanodrop system by measuring optical density at 260 nm.

2.1.11. Oligonucleotide primers design

Transcript sequences for reference gene and for the genes of interest were obtained from the National Centre for Biotechnology Information (NCBI,

http://www.ncbi.nlm.nih.gov/), and were cross referenced to the Ensembl canine genome data base (www.ensembl.org). Primer sequences were then designed by Primer3 (https://sourceforge.net/projects/primer3). BLAST searches were performed for all prime sequences to confirm gene specificity. All primers were synthesized by TIB-Molbiol (Berlin, Germany). An extensive set of experiments was done to optimize primer amplification. The description of the designed primers is shown in Table 1.

Table 1: Sequence and characteristics of various oligonucleotide primers

Gene	S/A	Primer sequences (5´-3´)	Location	Size (bp)	Accession No.
*IL-1β	S	CACAGTTCTCTGGTAGATGAGG	121-142	261	DQ251036
	A	TGGCTTATGTCCTGTAACTTGC	383-362		
*TNFα	S	CTCTTCTGCCTGCTGCAC	150-167	288	NM_001003244
	A	GCCCTTGAAGAGGACCTG	420-437		
*IL-1β	S	AGAGGATCCATGGCAGCAGTACCCGA	01-17	798	DQ251036
	A	AATGCGGCCGCCTAGGAAGAGAATTCCAT	798-781		
*TNFα	S	GCAAAGCTTAAGGACACCATGAGCACTGA	03-22	713	NM_001003244
		CCTAGAATTCTTACAGGGCAATGATTCC	697-716		
*IL-1β	S	ATAGGATCCGTGGACTGCAAGTTACAGGAC	355-375	444	DQ251036
	A	AATGCGGCCGCCTAGGAAGAGAATTCCAT	798-781		
*TNFα	S	CTACGGATCCATGAGTGACAAGCCAGTAGC	264-280	455	NM_001003244
	A	CCTAGAATTCTTACAGGGCAATGATTCC	697-716		
IL-1β	S	AGTTGCAAGTCTCCCACCAG	149-169	177	DQ251036
	A	TATCCGCATCTGTTTTGCAG	325-345		
IL-6	S	GGCTACTGCTTTCCCTACCC	108-128	198	NM_001003301
	A	TTTTCTGCCAGTGCCTCTTT	305-325		
IL-8	S	TCTTGGCAGCTTTTGTCCTT	32-52	151	NM_001003200
	A	GGGCCACTGTCAATCACTCT	182-202		
TNFα	S	TCATCTTCTCGAACCCCAAG	235-255	157	NM_001003244
	A	ACCCATCTGACGGCACTATC	391-411		
GM-CSF	S	CACTGTGGTCTGCAGCATCT	36-56	248	S49738
	A	GATTCTTGAGGCTGGTGAGG	283-303		
iNOS	S	GGAGGAGCAGCTACTGTTGG	1227-1246	178	AF068682
	A	GTCATGAGCAAAGGCACAGA	1385-1404		
COX-2	S	GCCTTACCCAGTTTGTGGAA	1239-1258	163	NM_001003354
	A	AGCCTAAAGCGTTTGCGATA	1382-1401		
MMP-3	S	CATTTATGGAGATGCCCACT	594-613	124	NM_001002967
	A	GTCAGCCGAGTGAAAGAGAC	698-717		
MMP-13	S	CAGACTTCATGACGGCACT	438-456	104	AF201729
	A	AAGCATGAGCCAGAAGACC	523-541		

(continued)

Gene	S/A	Primer sequences (5´-	Location	Size (bp)	Accession No.
Type I collagen	S	GAACCTGGCAAACAAGGTC	3017-3035	150	NM_001003090
	A	AGGAGAACCATCTCGTCCA	3148-3166		
Type II collagen	S	GAAACTCTGCCACCCTGAAT	3878-3897	160	NM_001006951
	A	GCTGCTCCACCAGTTCTTCT	4018-4037		
Aggrecan	S	CTATGAGGACGGCTTTCACC	573-592	194	U65989
	A	AGACCTCACCCTCCATCTCC	747-766		
CRTAC1	S	TTCTGCCCCCTGACTATGAC	249-268	171	XM_845961
	A	CATCCACTGCAATGTTCACC	400-419		
COMP	S	GAACCCAGACCAACGCAAT	1011-1029	221	XM_860228
	A	CATCTCCTACACCGTCACCA	1212-1231		
Sox9	S	CACCGAACAGACGCACAT	576-593	185	NM_001002978
	A	GCTTCAGGTCAGCCTTGC	743-760		
IL-4	S	CTCACCTCCCAACTGATTCC	70-89	156	NM_001003159
	A	CTTGACAGTCAGCTCCATGC	206-225		
STAT6	S	TTGGCTTCATCAGCAAACAG	1-20	228	EU439612
	A	GGTTTTTGAGCTGAGCAAGG	209-228		
*STAT6	S	TTGGCTTCATCAGCAAACAG	1-20	920	EU439612
	A	CATCGAATTCTCACCAGCTAGGGTTGG	904-920		
GAPDH	S	TAT TGT CGC CAT CAA TGA CC	81-100	195	NM_01003142
	A	TAC TCA GCA CCA GCA TCA CC	261-275		
β-actin	S	GGCATCCTGACCCTCAAGTA	123-142	215	Z70044
	A	ACATACATGGCTGGGGTGTT	318-334		
18s rRNA	S	GGATGCGTGCATTTATCAGA	202-221	244	DQ287955
	A	GTTTCTCAGGCTCCCTCTCC	426-445		

*primers used for cloning purpose; S = sense (forward); A = antisense (reverse); bp = base pairs

2.1.12. Reverse transcription polymerase chain reaction (RT-PCR)

PCR was carried out to obtain canine type I collagen, type II collagen, aggrecan, CRTAC1, COMP and Sox9. The gene for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as endogenous reference. GAPDH was chosen as the reference housekeeping gene based on the results obtained by comparing it with β-actin and 18s rRNA. GAPDH showed minimum variation in the levels of transcription of respective mRNA between samples taken at different time points. The PCR reaction comprised of to a final concentration of 1x NH_4 buffer, 1.5 mM $MgCl_2$, 200 µM four dNTP mix, 0.1 µM sequence specific primers and 5 U of Mango DNA Taq Polymerase in a total 50 µl master mix. Amplification was carried out in Mastercycler gradient. PCR conditions after initial denaturation at 95^{o}C for 2 min were: denaturation at 95^{o}C for 30 sec; annealing at 55^{o}C for 20 sec; extension at 72^{o}C for 30 sec for 35 cycles, followed by final extension at 72^{o}C for 10 min.

2.1.13. Agarose gel electrophoresis

PCR products and DNA molecular weight marker were separated by electrophoresis (1-5 volts/cm for 20-25 min) on a 2% Tris-acetate-EDTA (TAE) agarose gel with 0.5 µg ml^{-1} ethidium bromide. The DNA bands were visualized using UV transilluminator and digital photographs were documented.

2.1.14. Quantitative real time PCR (qRT-PCR)

The expression profile for multiple chondrocyte marker genes (mentioned above) was measured using qRT-PCR on an iCycler iQ5. GAPDH was used as endogenous reference for normalization of threshold cycles (C_t) values of target genes. PCR reactions were prepared under a sterile UV hood with air circulation. Only sterile, disposable reaction tubes and filter tips were used and at all steps disposable powder-free gloves were worn. PCR reactions were carried out using a 20 µl reaction volume (in 96 well plate sealed with film) containing 10 µl of the SensiMix plus SYBR with fluorescein containing 3 mM $MgCl_2$. The primers were used at 500 nM and the cDNA was added at the concentration of 500 ng. Each sample was analyzed in triplicate. PCR conditions after initial denaturation at 95^{o}C for 3 min were: denaturation at 95^{o}C for 20 sec; annealing at 55^{o}C for 20 sec; extension at 72^{o}C for 20 sec for 35 cycles. The green fluorescence was measured at 95^{o}C for 30 sec and finally at 55^{o}C for 10 sec for 41 cycles. The C_t values were determined automatically by the optical system software version 2.0.

2.1.15. Calculation of differential index

‘‘Differentiation index’’ was defined with the ratios of mRNA levels of type II collagen to type I collagen (CII/CI) using real time data. Final numeric values were calculated and expressed in arbitrary units (Martin *et al.,* 2001; Marlovits *et al.,* 2004)-.

2.1.16. Western blot analysis for collagen expression

Expression of type I collagen and type II collagen in chondrocytes was also analyzed by Western blot as described by Salvat *et al.* (2005). Protein was isolated from chondrocytes at P1-P7 using TRIsure reagent (Bioline, Germany) according to manufacturer’s instructions. To the protein samples 1 mM PMSF (Phenylmethylsulphonyl fluoride) as proteinase inhibitor was added and stored at -20°C until used. The protein concentration was determined (Lowry *et al.,* 1951) with minor modifications using bovine serum albumin (BSA) as the standard. The absorbance was measured on a TECAN spectra ELISA reader at 750 nm wavelength.

For Western blot analysis, 35 µg protein samples were size-fractionated by 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described (Laemmli, 1970). Proteins were transferred electrophoretically to polyvinylidene difluoride (PVDF) membrane for 90 min at 110 mA (1 mA/cm^2). Blots were blocked for 1 h with blocking buffer. The membranes were then incubated for overnight with goat anti-collagen type I polyclonal or rabbit anti bovine type II collagen polyclonal at 1:500 dilutions in blocking buffer at RT. After washing three times for 10 min with blocking buffer, membranes were incubated for 1 h with following secondary antibodies: anti-goat horseradish peroxidase conjugated diluted 1:8000 (for type I collagen) or anti rabbit horseradish peroxidase conjugated diluted 1:10000 (for type II collagen) in blocking buffer for 1 h at RT. The membranes were then washed four times for 10 min in the blocking buffer. The blot was developed using an enhanced chemiluminescence (CLS) system according to the manufacturer’s instructions and subsequently exposed to X-ray film to visualize protein bands.

2.1.17. Generation of chondrocyte cell line

Taking into consideration the limited proliferation ability of primary chondrocytes, attempts were made to generate a stable canine chondrocyte cell line. This was

aimed to gain indefinite proliferation capacity of chondrocytes for use in the development and characterization of inflammation model *in vitro*.

2.1.18. DNA source and preparation

hTERT expression construct cloned in pCI.neo vector (pCIneo-hTERT) was a generous gift from Dr. Lorel Colgin, Children's Medical Research Institute (CMRI) Sydney, Australia. The activity of the clone was tested by telomerase repeat amplification protocol (TRAP) assay before it was shipped (Colgin *et al.,* 2000).

2.1.19. Preparation of competent cells

XL1-Blue cells were made competent by using the $CaCl_2$ method as described elsewhere (Sambrook *et al*., 1989). From the frozen stock, bacterial cells were picked up by the pipette tip and incubated in 3 ml YT medium at 37^{O}C for overnight on constant shaking. The fresh culture was then diluted to 1:100 in YT medium and allowed to grow until logarithmic growth phase (OD_{600}) reached 0.5. Cells from 100 ml culture were then harvested by centrifugation at 1000xg for 10 min. the pellet was resuspended in 25 ml ice cold $CaCl_2$ solution (50 mM $CaCl_2$, 10 mM Tris HCl, pH 8.0) and incubated on ice for 30 min followed by centrifugation. The cells were then resuspended in 5 ml ice cold $CaCl_2$ solution and incubated overnight at 4^{O}C. To this bacterial cell suspension 1.25 ml of 80% glycerol was added and mixed by inverting. Finally, the cells were aliquoted in 0.6 ml volume in sterile cryotubes, chilled in liquid nitrogen and stored at -80^{O}C.

DH5α and BL21(DE3)p(Lys) cells were made competent by using Z-competent *E. coli* transformation buffer set according to manufacturer's instructions.

2.1.20. Transformation of competent cells and colony screening

The plasmid DNA was transformed into competent high *E. coli* XL1-Blue cells using heat shock. Briefly, 200 µl of competent cells were thawed on ice and 2 µg of plasmid DNA was gently mixed. The cells-DNA mixture was incubated on ice for 30 min followed by heat shocking at 42^{o}C for 45 sec and further incubation on ice for 2 min. After adding 500 µl Yeast extract Tryptone (2-YT broth) medium the cells were incubated at 37^{o}C for 1 h with constant shaking. The mixture was centrifuged at 6000 rpm for 3 min and the supernatant about 500 µl was removed and the pellet was resuspended in the remaining 200 µl of the supernatant. The resuspended pellet plated onto YT agar plate with 100 µg ml^{-1} ampicillin followed by an overnight incubation at 37°C. Next day, few randomly selected colonies were inoculated in 5 ml

YT medium containing 100 µg ml^{-1} ampicillin in culture tubes and incubated overnight at 37^{o}C for propagation.

2.1.21. Mini-plasmid preparation

The mini-scale preparation of pure plasmid DNA from bacterial cultures was obtained using a spin plasmid mini-kit (Invitek, Germany) according to manufacture's instructions. Concentration and purity of DNA was determined by Nanodrop system.

2.1.22. Restriction enzyme digestion

The plasmid DNA has telomerase catalytic subunit hEST2 (human Ever Shorter Telomeres 2) sequence (position 51-3456) inserted into pCI-neo mammalian expression vector at the EcoRI and SalI sites. Therefore, insert size was confirmed by these restriction endonucleases. In a 20 µl reaction volume 1 µg plasmid DNA was incubated with 2 µl of each EcoRI and SalI and 2 µl of 10x NEBuffer EcoRI and 2 µl of 10x BSA. The reaction mixture was incubated at 37^{o}C for 90 min. The digested product along with undigested control was analyzed by a 1.5% TAE agarose gel.

2.1.23. DNA Sequencing

Two micrograms of the plasmid DNA in 20 µl aqua dest were commissioned to commercial laboratory (Services in Molecular Biology, Berlin, Germany) for DNA sequencing. The sequence data files were read using Chromas software (www.technelysium.com.au/chromas.html). Sequence homology by nucleotide and predicted amino acids of the hTERT gene fragments were determined using the Basic Local Alignment Search Tool (BLAST) and FASTA format (DNA Data Bank of GenBank (NCBI), Bethesda MD, USA; www.ncbi.nlm.nih.gov).

2.1.24. Transfection and selection of chondrocytes

Primary chondrocytes were transfected using electroporation by Amaxa nucleofector. Briefly, immediately before transfection chondrocytes were trypsinized, washed with PBS, and then resuspended in Nucleofector solution (for human chondrocytes) containing 2 µg of pCIneo-hTERT plasmid to a final concentration of $1x10^{6}$ cells/100µl. Positive control vector 2µg pmaxGFP encoding green fluorescent protein (GFP) was used. Samples were subjected to nucleofection using Amaxa nucleofector devices at settings recommended by manufacturer for primary human chondrocytes (program U-24). After transfections cells were cultured inn pre-warmed growth medium. Selection of resistant clones (containing hTERT plasmid) with genticin

sulphate (G418) was started 5 days after transfection. Medium containing G418 (400 µg ml^{-1}) was changed three times a week for the first two weeks after which the concentration of G418 was reduced to five times (80 µg ml^{-1}). This concentration was maintained during and after G418-resitant colonies appeared in approximately 4 w and positive clones were selected, pooled and named as GD-hTERT. Growth kinetics, proliferation and phenotype modulations were monitored in 2D cultures. Immortalized chondrocytes were then subjected to 3D culture system for re-differentiation.

2.1.25. Characterization of immortalized chondrocytes

Immortalized GD-hTERT cells were monitored for growth rate and morphology for up to P20. The cells were monitored on daily basis to record their phenotype and were photographed at each passage in 2D. Molecular and phenotypic modulations of immortalized cells were determined in 2D and 3D cultures. The expression of multiple chondrocyte marker genes was quantified by qRT-PCR.

2.2. Characterization of *in vitro* 3D model of inflammatory arthritis

To establish an *in vitro* model of inflammatory arthritis in chondrocytes, species specific pro-inflammatory cytokines, canine IL-1β and canine TNFα were amplified by RT-PCR from canine blood. These PCR products were cloned and expressed in bacterial expression vector to obtain recombinant proteins which were used as stimulants in chondrocytes.

2.2.1. Cell isolation and *in vitro* culture

Canine whole blood samples were obtained from Clinic for Small Animals, Freie Universität Berlin. Peripheral blood mononuclear cells (PBMC) were prepared from peripheral blood by Ficoll-Plaque plus (Amersham Biosciences, USA) density gradient centrifugation according to the manufacturer's instructions (Liu *et al.,* 2005). The obtained PBMC were counted and cultured at a density of $1x10^6$ cells in 35 mm cell in RPMI 1640 medium with 10% FCS and 1% penicillin/streptomycin. The cells were incubated at 37^oC with 5% CO_2 for 24 h.

2.2.2. Cell stimulation

To stimulate the expression of IL-1β and TNFα cultured PBMCs were incubated with (treatment) or without (control) commercially available human recombinant IL-1β and/or TNFα proteins at a concentration of 100 ng ml^{-1} in RPMI 1640 medium with 1% FCS. After 72 h of incubation the cells were trypsinized and centrifuged at 200xg for 10 min. The obtained cell pellet was washed twice with PBS and finally kept at -80^oC until used for total RNA extraction.

2.2.3. RT-PCR amplification and nested PCR

Total RNA was extracted from the cell pellet using RNeasy plus mini kit (Qiagen, Germany) according to the manufacturer's instructions. RNA was subjected to DNase degradation before processed for cDNA synthesis. To synthesize IL-1β and TNFα genes RT-PCR was carried out. PCR conditions after initial denaturation at 94^oC for 2 min were: denaturation at 94^oC for 30 sec; annealing at 55^oC for 30 sec; extension at 72^oC for 90 sec for 30 cycles, followed by final extension at 72^oC for 10 min. The PCR products and molecular weight marker were separated by electrophoresis on a 1.5% TAE agarose gel. The desired band of amplicons was excised with a scalpel and transferred to a microfuge tube and weighed. This product was then purified from agarose gel fragment by Jetsorb gel extraction kit (Genomed, Germany) according to manufacturer's instructions. Two micrograms of purified PCR product were used to

confirm the DNA by nested PCR using sequence specific internal primers. The PCR reaction mixture was set as described above except that the extension time was reduced to 30 sec for a total 25 cycles. The PCR products were analyzed by electrophoresis on a 2% TAE agarose gel.

2.2.4. Ligation and transformation of DNA

Pure PCR product was ligated as an insert into a T-overhang vector pGEM-T. In a total 20 µl reaction volume 1 µl of insert (PCR pure product) was mixed with equal volume (1:1 molar ratio) of vector using 0.3 Weiss units μl^{-1} T4 DNA ligase with 2 µl of 10x T4 DNA ligase buffer in nuclease free water. The ligation mixture was incubated at 16^{o}C for 16 h and then inactivated by heating at 65^{o}C for 15 min. Ligation product was stored at –20^{o}C until transformed into bacteria. The ligation mixture was transformed into competent high *E. coli* DH5α cells using heat shock on agar plates with ampicillin (100 µg ml^{-1}).

2.2.5. Colony screening

After 24 h, some of the colonies from the plate were randomly selected and were subjected to colony PCR to confirm the presence of the cloned insert by amplification. Briefly, a small fragment of the colony was mixed in a PCR reaction. The positive colonies were inoculated in 5 ml YT medium with 100 µg ml^{-1} ampicillin in culture tubes and incubated overnight at 37^{o}C for propagation or mini-plasmid preparation.

2.2.6. Mini-plasmid preparation

The mini-scale preparation of plasmid DNA from bacterial cultures was done using a modification of a method previously reported (Del Sal *et al.,* 1988). In short, 1.5 ml of overnight bacterial culture was taken in a microfuge tube and centrifuged at 13000 rpm for 5 min. The supernatant was discarded, pellet resuspended in 200 µl STET buffer along with 4 µl lysozyme (50 mg ml^{-1}) and incubated at RT for 5 min followed by boiling for 45 sec in a water bath. The boiled mixture was then centrifuged at 13000 rpm for 10 min. The slimy pellet was removed with a sterile toothpick and to the supernatant 8 µl of pre-warmed CTAB (5% w/v) was added. The precipitate was centrifuged at 13000 rpm for 5 min, the supernatant discarded and the pellet resuspended in 300 µl of 1.2 M NaCl solution through extensive vortexing. DNA was then precipitated with ice-cold absolute ethanol and centrifuged for 10 min followed by washing in 70% ice-cold ethanol. DNA pellet was air dried and resuspended in 30

µl of TE buffer (pH 8.0) containing RNase A (20 µg ml^{-1}). The eluted DNA was placed on ice until used for restriction analysis or PCR and stored at –20^{o}C.

2.2.7. Restriction enzyme analysis

The resulting plasmid was verified by restriction enzyme digestion. The sense and antisense primers were designed with restriction sites for BamHI and NotI for IL-1β. In a 20 µl reaction volume 1 µg of DNA (pGEMT-IL-1β) was incubated with 2 µl (40 units) of each BamHI and NotI with 2 µl each of 10x NEBuffer 3 and 10x BSA. Similarly, sense and antisense primers for TNFα had restriction sites for HindIII and EcoRI. In 20 µl reaction volume 1 µg of DNA (pGEMT-TNFα) was incubated with 2 µl (40 units) of each HindIII and EcoRI with 2 µl of 10x NEBuffer EcoRI. The reaction mixtures were incubated at 37^{o}C for 90 min. After incubation the digested products along with undigested control were analyzed by electrophoresis on a 1% TAE agarose gels.

2.2.8. Maxi-plasmid preparation

The plasmid DNA for both cytokines was subjected to more purified and concentrated high copy plasmid by maxi-plasmid preparation (Qiagen, Germany) according to supplied manual. Purity and concentrations of plasmid DNA was determined and presence of respective inserts was confirmed by restriction endonucleases and by sequence analysis. The multiple sequence alignments were made by ClustalW, an online EBI web server at www.ebi.ac.uk/clustalW.

2.2.9. Sub-cloning for eukaryotic expression

Recombinant IL-1β and TNFα were sub-cloned in DsRed1-N1 and pcDNA3.1 vectors respectively for eukaryotic expression studies. For this purpose the pGEMT-IL-1β and DsRed1-N1 vector were digested separately with restriction endonucleases, BamHI and Not I and pGEMT-TNFα as well as pcDNA3.1 vector were digested using endonucleases HindIII and EcoRI. The digested and undigested control samples were run parallel on 1% agarose gel, desired fragments were confirmed by comparing size with DNA marker and cut from the gel and purified. The purified set of inserts and vectors were ligated and 5 µl of ligation mixture was transformed in competent DH5α cells by heat shock. The transformed bacteria were plated on YT-agar with kanamycin (25 µg ml^{-1}) for DsRed1N1-IL-1β and ampicillin (100 µg ml^{-1}) for pcDNA3.1-TNFα. The plates were incubated for overnight at 37^{o}C and resulting colonies were grown in YT medium with respective antibiotics for mini-plasmid

preparation. The DNA for respective clones were reconfirmed by restriction digestion and subjected to more purified and concentrated high copy plasmid by maxi-plasmid preparation.

2.2.10. Cloning in bacterial expression vector

Polymerase chain reaction was carried out to amplify IL-1β and TNFα without signal peptide using sequence specific primers designed for expression of the gene products as His-tag fusion proteins. The PCR was carried out with IL-1β-MP or TNFα-MP primers using pGEMT-IL-1β or pGEMT-TNFα as DNA templates. The obtained amplicons were excised, purified and subjected to digestion using enzymes BamHI and EcoRI for both IL-1β and TNFα along with bacterial expression vector pRSET-A. This vector for an N-terminal detection and purification epitope (6X His-tagged) as specified by the manufacturer. The digested and purified products were ligated and transformed in DH5α competent cells using YT medium with ampicillin (100 μg ml^{-1}). The cloned genes were confirmed following mini-plasmid preparation by restriction enzyme digestion and finally sent for sequencing.

2.2.11. Bacterial expression of recombinant cytokines

To express pRSETA-IL-1β or pRSETA-TNFα, they were transformed into the BL21(DE3)p(Lys) strain of *E. coli*. The single colony of transformed *E. coli* was picked out and inoculated in a tube containing 5 ml YT medium supplemented with ampicillin (100 μg ml^{-1}) and cultured at 37°C until cell density (OD_{600}) reached 0.6-0.7. The culture was induced by addition of the final concentration of 1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) for overnight at 37°C. The proteins were analyzed by SDS-PAGE. One-third volume of 3x non-reducing sample buffer was added to the samples before incubation at 95°C for 5 min followed by centrifugation at 14000 rpm for 5 min. The samples were separated on 15% gel by running parallel the pre-stained protein marker. The gel was stained by 0.5% Coomassie brilliant blue-R250 dye for 45 min and finally de-stained with 10% acetic acid and 10% methanol until the background became clear. Finally the gel was dried under vacuum at 80°C for 95 min using a gel dryer and then scanned.

2.2.12. Purification of recombinant proteins

For purification, we used Ni-NTA slurry (Qiagen, Germany) according to the manufacturer's instructions with minor modifications. Briefly, The pRSETA-IL-1β and pRSETA-TNFα cloned from *E. coli* strain BL21(DE3)p(Lys) were grown at 37°C in YT

medium (100 µg ml^{-1} ampicillin) to the optimal density of 0.6 (600 nm). Then, IPTG (Isopropyl β-D-1-thiogalactopyranoside) to final concentration of 1 mM was added to induce protein production for 4 h at 30^{o}C. The cells were harvested by centrifugation at RT, dissolved in lysis buffer and incubated on ice for 1 h. The suspension was sonicated with a sonicator (six times for 10 sec each with 5 sec pauses between) on ice and centrifuged to remove insoluble materials. Expression of recombinant protein was detected by 15% SDS-PAGE. Small-scale expression revealed that recombinant IL-1β and TNFα were produced in the cytosolic fraction. Proteins were purified using Ni-NTA agarose affinity column. The column was washed extensively with wash buffer (1M NaCl, 50 mM Tris (pH 7.6), 20 mM imidazole and 0.5% triton X-114) to remove bacterial proteins. Finally recombinant proteins were eluted with elution buffer (50 mM Tris (pH 7.6), 150 mM NaCl, and 300 mM imidazole). The purified proteins were resolved on 15% SDS-PAGE and were detected by Coomassie blue and silver staining or by Western blot as described below.

2.2.13. SDS-PAGE and silver staining

Extracts and column fractions from affinity purification were analyzed by 15% SDS-PAGE gels and recombinant proteins were visualized initially by staining with Coomassie blue and subsequently by silver. Silver staining was performed similarly to the method described (Rabilloud *et al.,* 1988) with slight modifications. After electrophoresis, the gel slab was fixed in fixation solution for 1 h. It was then washed twice for 20 min with 50% ethanol in water and additionally for 10 min with water to remove the remaining acid. The gel was sensitized by incubation in reducer (0.02% sodium thiosulfate) for 1 min and was then rinsed with three changes of distilled water for 20 sec each. After rinsing, the gel was submerged in 0.16% silver nitrate solution and incubated for 30 min. After incubation, the silver nitrate was discarded, and the gel slab was rinsed twice with water for 20 sec and then developed in developer with intensive shaking. After the desired intensity of staining was achieved, the development was terminated by 1% glycin, followed by washing of the gel slab with water and then kept in 10% ethanol and 2% glycerol solution until analyzed. The approximate molecular weights of IL-1β and TNFα were estimated by interpolation of distance migrated by protein markers of lesser and greater molecular weight than the recombinant proteins. The theoretical molecular weights of IL-1β and TNFα were determined using an online protein calculator v3.3 (http://www.scripps.edu/~cdputnam/protcalc.html).

2.2.14. Western blotting for His-tag protein

To confirm the molecular mass of recombinant cytokines we performed Western blot analysis of IL-1β and TNFα histidine fusion protein. The concentration of recombinant proteins was measured by Lowery assay before loading on SDS gels for Western blot. The proteins in serial dilutions (200 ng, 100 ng, 50 ng and 25 ng) were loaded on 15% SDS gels. As a positive control we used *E. coli* expressed IL-4 protein (50 ng) previously cloned and purified in this lab (Wondimu *et al.,* 2001). Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane by electro-blotting (1 mA/cm^2). Anti His-tag monoclonal antibody (conjugated with HRPO) diluted 1:2000 in PBS-T was used to detect the protein.

2.2.15. Endotoxin test

The endotoxin content of different fractions was determined by the Limulus amoebocyte lysate (LAL) kinetic-QCL assay according to the manufacturer's instructions, (Cambrex Bioscience, USA). Values were expressed as EU ml^{-1} (Zimmerman *et al.,* 2006).

2.2.16. Stimulation of chondrocytes with cytokines

A total of $5x10^5$ cells were cultured in each well of 6-well plates with growth medium for 2D inflammation model. Likewise, cells were encapsulated in alginate beads and approximately 10-12 beads were cultured in 6-well plates with growth medium. After 24 h the cells/beads were washed twice and medium was replaced to 1% FCS. The cells in 2D as well as in alginate beads were then stimulated with 100 ng ml^{-1} of IL-1β and 50 ng ml^{-1} TNFα proteins alone or in combination. As a control, cells or beads were treated with fractions of a mock purified protein from *E. coli* transformed with an empty vector. Supernatants were collected and cells were harvested from 2D and 3D cultures at 24, 48 and 72 h post-stimulation.

2.2.17. Quantification of inflammation markers

Gene expression levels for canine cytokines (IL-1β, TNFα, IL-6, IL-8, GM-CSF) enzyme mediators (iNOS, COX-2, MMP-3, MMP-13), aggrecan and type II collagen were quantified by qRT-PCR.

2.2.18. Nitrite determination

Supernatants from cultured chondrocytes were aspirated and stored at –20°C until assayed. Concentration of nitrite, a stable product of NO was measured using

calorimetric assay based upon Griess reagent system (Promega, Germany). Results were expressed as µmol L^{-1} nitrite (Green *et al*., 1982).

2.2.19. PGE_2 determination

Aliquots of supernatants from stimulated and non-stimulated chondrocytes were collected and the PGE_2 concentrations were measured using a commercially available PGE_2 enzyme immunoassay kit (Cayman Chemical Co., USA) according to manufacturer's instructions. Results were expressed in pg ml^{-1} PGE_2 (Masuko-Hongo *et al*., 2004).

2.3. IL-4 transgene expression in chondrocytes

2.3.1 Transfection of chondrocytes

Chondrocytes were seeded at a density of 1x 10^6 in 6 cm Petri dishes in complete medium for 24 h. Cells were washed twice with PBS and incubated with DMEM containing 1% FCS. Chondrocytes were transfected using the nonliposomal lipid formulation FuGENE 6 as previously described (Madry *et al.,* 2003). Briefly, cells were cultured in 6 cm tissue culture dishes in growth medium and allowed to grow under standard incubation conditions. After the cells reached sub-confluence, medium containing 1% FCS was added. Cells were treated with 4 U ml^{-1} bovine testicular hyaluronidase 6 h before and during transfection. DNA amounts and FuGENE 6/DNA ratios were determined to optimize the contribution of DNA dose and FuGENE 6/DNA ratio to transfection efficiency. A total of 8 µg of endotoxin-free plasmid DNA (pCDNA3.1-IL-4) complexed with 12 µl FuGENE 6 (FuGENE 6/DNA ratio 3:2) in basal medium and transferred to subconfluent chondrocyte monolayers and were incubated under standard incubation conditions. As a control empty plasmid vector (pCDNA3.1) was used for transfection.

2.3.2 Protein sample preparation

The supernatant was taken from the cells 72 h post-transfection and cells were collected by centrifugation and lysed using RIPA buffer containing 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 0.15 M NaCl, 20 mM Tris, 10 mM EDTA, 10 mM iodoacetamide, 1 mM PMSF) supplemented with protease inhibitors (10 µg ml^{-1} each of aprotinin, leupeptin and pepstatin). After 30-min incubation on ice, lysates were centrifuged at 10,000*g* for 15 min at 4°C. Lysate was quantified for protein concentration by Lowry micro-assay.

The cell supernatants were concentrated using Chloroform: methanol method as described (Wessel and Flügge, 1984). To 1 ml of supernatant 4 ml of methanol was added mixed well with the supernatant followed by addition of 1 ml of chloroform and 3 ml of water. The reaction was mixed vigorously by vortexing, centrifuged at 9000xg for 2 min and the upper phase was discarded. Then 3 ml of methanol was added to lower phase and interphase with precipitated protein, mixed and centrifuged at 9000xg for 2 min to pellet protein. The pellet was washed once with 80% methanol and set to air dry. The protein was eluted in 100 µl of elution buffer and proteinase inhibitors were added. Protein concentration was measured and kept at -20^oC until used.

2.3.3 Western blot analysis

A total of 20 µg of protein was mixed with loading buffer containing SDS and 2-ME, boiled for 5 min and separated on 15% SDS-PAGE gradient gels. Proteins were electro-transferred onto PVDF membrane (Pierce, USA) using 1 mA/cm^2 for 90 min, with constant voltage. Membrane was blocked in 5% nonfat dry milk in PBS and 0.5% (v/v) Tween 20. IL-4 protein was detected with rabbit anti-canine-IL-4 (1:1000 dilution) as primary antibody and mouse anti-rabbit horseradish peroxidase conjugated as a secondary antibody (Sigma, Germany). Blots were developed with SuperSignal West Pico chemiluminescent substrate and CL-Xpose film.

2.3.4 Enzyme linked immunosorbent assay (ELISA)

To measure the amount of IL-4 protein concentrated cell supernatant and cell lysate subjected to sandwich ELISA based on a standard protocol developed in this Lab. Briefly, 96-well plate. was coated with rabbit anti-IL-4 polyclonal antibody (2.4 mg ml^{-1}; 1:1000 dilution) in carbonate buffer (pH 9.6) for 1 h at RT. Plate was blocked with 1% BSA dissolved in PBS for 2 h at RT and wells were rinsed with PBS. The standards (*E. coli* expressed recombinant IL-4 protein) were serially diluted (1 ng protein as starting material) in blocking buffer and were dispensed in triplicates in lower wells of 96-well plate. Similarly, test samples (concentrated cell culture supernatants and cell lysates) were also diluted in blocking buffer and dispensed in separate wells in triplicates. The plate was incubated for 1 h at RT and was then washed with PBS containing 0.1% Tween-20. After washing, secondary antibody (anti mouse IL-4 monoclonal) was added at (1:10 dilution) in carbonate buffer and incubated for 1-2 h at RT. Wells were rinsed with PBS and then samples were incubated with anti-mouse biotinylated antibody (dilution 1:2500) for 30 min at RT followed by washing with PBS. Afterwards the samples were incubated with HRP-conjugated streptavidin for 30 min (dilution 1:4000). Finally chromogenic substrate *o*phenylenediamine (OPDA) with hydrogen peroxide (30%) was given to each well of the plate and incubated in dark for 20-30 min. Colour development was measured at 492 nm in an ELISA reader after stopping the reaction with 1M sulphuric acid. In this experiment, the washing was carried out in an ELISA washer.

2.3.5 Chondrocyte stimulation

Chondrocytes were transfected with pcDNA3.1-IL-4 or with empty plasmid (mock transfection) using FuGENE 6 as described above. Cells were either kept monolayer or transferred to alginate beads 24 h post-transfection. Cells or beads were

stimulated IL-1β (100 ng ml^{-1}) and TNFα (50 ng ml^{-1}) as before. As a control, chondrocytes without any stimulation were used. Supernatants were collected and cells were harvested 48 h post-stimulation (72 h post-transfection). The cell supernatants were used for nitrite and PGE_2 assays, and the cells were pelleted for total RNA isolation.

2.3.6 Quantification of inflammation markers

Gene expression levels for inflammatory mediators (IL-1β, TNFα, IL-6, IL-8, iNOS) were quantified by qRT-PCR. Nitrite and PGE2 concentration were determined in cell culture supernatant using respective kits as described above.

2.3.7 Expression of IL-4 and STAT6

The expression of IL-4 transgene and STAT6 was quantified by qRT-PCR. STAT6 was amplified from IL-4 transduced chondrocytes by PCR. The fragment was cloned into T-overhang vector and sent for sequencing.

2.3.8 Cloning and sequencing of partial STAT6 cDNA

Another important measure of the IL-4 expression is the expression of STAT6 which is involved in the anti-inflammatory pathway of IL-4. Since canine STAT6 has not yet been published, efforts were made to clone canine STAT6. Primers were designed from the predicted sequence (Accession No. BK006461) and RT-PCR was used to amplify a fragment of canine STAT6 from pcDNA3.1-IL-4 transfected cells. The cDNA was cloned in pGEM-T vector and submitted for sequence analysis.

2.3.9 Statistical analysis

Results are representative of three independent experiments. All values are presented as mean ± S.E.M.

2.4. Materials

2.4.1. Animal (dog) source

A total of 5 dogs were included in this study and 9 joint samples were taken. Dogs for this study were selected from those routinely euthanized at the Clinic for Small Animals of the Free University Berlin, Germany. Knee joints were surgically removed for cartilage tissue collection. Table 2 summarizes the data of dogs used for these experiments.

Table 2: Preliminary data representing breeds of dog, age, sex, cartilage source and cartilage wet weight

No.	*Breed*	*Alias*	*Age*	*Sex*	*Cartilage*	
					Source	Weight
1.	Russian Terrier	R-T	7 years	Male	Stifle joint	1.5 g
2.	Great Dane	GD	6 Months	Male	Stifle joint	3.2 g
3.	Shar Pei	SP	2 years	Female	Stifle joint	0.3 g
4.	Labrador	LD	4 Months	Male	Stifle Joint	2.0 g
5.	Weimaraner Welpen	WW	3 Months	Male	Stifle Joint	3.6 g

2.4.2. Plastic/Glassware, chemicals and disposables

Plastic-ware were obtained from greine bio-one (Frickenhausen, Germany) unless otherwise indicated. Chemicals were either purchase from Sigma-Aldrich (Taufkirchen, Germany) or Carl Roth (Karlösruhe Germany). Glassware and disposable were supplied by different manufacturers

2.4.3. Materials for cell culture

RPMI 1640; PBS; HANKS' solution; Trypsin-EDTA	Biochrom AG, Seromed, Berlin, Germany
DMEM	Pan Biotech, Aidenbach, Germany
FCS	BioWhittaker Germany
Hyaluronidase; Collagenase P	Roche Diagnostics, Mannheim, Germany
Collagenase type CLS	Biochrom AG, Seromed, Berlin, Germany

2.4.4. RT-PCR/ligation reaction/endonucleases

RT-PCR (cDNA synthesis)	Fermentas, St. Leon-Rot, Germany
PCR reaction components	Bioline, Luckenwalde Germany
Ligation reagents	Promega, Mannheim, Germany
Restriction endonucleases	New England Biolabs, Taunus, Germany

2.4.5. Vectors

DsRed1-N1	Clontech Laboratories, Mountain View CA, USA
pcDNA3.1 pRSET-A	Invitrogen, Karlsruhe, Germany
pGEM-T	Promega, Mannheim, Germany
pmaxGFP	Amaxa Biosystems, Cologne, Germany

2.4.6. Competent cells

BL21(DE3)p(Lys)	Novagene (Merck) Darmstadt, Germany
DH5α	Invitrogen, Karlsruhe, Germany
XL1-Blue	Stratagene La Jolla, CA, USA

2.4.7. Antibiotics

Ampicillin Kanamycin	Carl Roth GmbH, Karlsruhe, Germany
Penicillin/ Streptomycin	Pan Biotech, Aidenbach, Germany
G418 Disulphate	AppliChem GmbH, Darmstadt, Germany

2.4.8. Transfection reagents

FuGENE 6	Roche Diagnostic, Mannheim, Germany
Nucleofector solution	Amaxa Biosystems, Cologne, Germany

2.4.9. Recombinant proteins

Human recombinant IL-1β; TNFα proteins	Sigma-Aldrich (Taufkirchen, Germany

2.4.10. DNA/protein markers

DNA λ Eco91 (Bst E II); O'RangeRuler 100 bp	Fermentas, St. Leon-Rot, Germany
HyperLadder IV DNA	Bioline, Luckenwalde, Germany
Pre-stained marker III	AppliChem GmbH, Darmstadt, Germany

2.4.11. Antibodies

Goat anti-IL-4	R&D Systems, Wiesbaden-Nordenstadt, Germany
goat anti-collagen type I	Chemicon International, Hofheim, Germany
Rabbit anti-bovine type II collagen	

2.4.12. Reagents

APS; TEMED	National Diagnostics, München
IPTG; Imidazol	Merck, Darmstadt, Germany
PMSF; Non-fat dry milk	AppliChem GmbH, Darmstadt, Germany
Tween 80	SERVA Feinbiochemica, Heidelberg, Germany
Acrylamide,	Carl Roth, Karlsruhe, Germany

Bisacrylamide; Ethidium bromide, OPDA, alcian blue	
Nickel – NTA – Agarose	QIAGEN, Hilden, Germany
Triton X 100; Triton X 114	Boehringer Mannheim, Mannheim
2-YT broth; Select agar	Gibco products, Invitrogen
FACS flow sheath fluid	BD Biosciences, Heidelberg, Germany
Ficoll-Plaque plus	Amersham Biosciences

2.4.13. Miscellaneous solutions/buffer

Elution buffer Chloroform: methanol	10 mM Tris-HCl, pH 6.8 + 0.5% SDS + 1 mM EDTA
6x loading buffer	4 g sucrose, 25 mg bromophenol blue, 2.4 ml of 0.5M EDTA (for agarose gel) 10 ml volume
Alginate dissolving solution	NaCl, sodium citrate pH 6.8
TE buffer	10 mM Tris HCl pH 8.0, 1 mM EDTA
50x Tris – Acetate – EDTA – buffer (1L)	242 g Tris pH 8.5 57.1 ml Vinegar 100 ml 0-5M EDTA

2.4.14. Miscellaneous items

Chamber slides 8 chamber polystyrene vessel	Falcon, Becton Dickinson Labware, Claix, France
CL–X Posure Film,	Pierce Biotechnology, Jena
ELISA plates 96-well flat bottom	NUNC Labormaterieal, Wiesbaden
Cell strainer (100 µm)	Falcon, Becton Dickinson Labware, Claix, France
PVDF–Transfer Membrane	Pierce Biotechnology, Jena, Germany
Real time PCR 96-well plates	Bio–Rad Laboratories, München, Germany
Spinner flasks	Wheaton, Science Products, NJ, USA
Sterile filter (0.22 µm)	Millipore S.A. Molsheim, France

2.4.15. SDS polyacrylamide mini-gels

DS-sample buffer	0.0625 M Tris, 2% SDS, 10% glycerin – non reducing 0.01% bromophenol blue, 5% β-Mercaptoethanol
Coomassie blue staining solution	0.3% (w/v) Coomassie brilliant blue G-250, 10% glacial acetic acid, 45% ethanol, adjust volume with water

De-staining solution	10% (v/v) glacial acetic acid, 10% (v/v) ethanol, adjust volume be distilled water

Table 3: Formulations for the preparation of mini-gels

Running gel	Volume for different percentages of acrylmide	
Components	7.5%	15%
Resolving gel 4X buffer	3.75 ml	7.5 ml
Water	3.75 ml	7.5 ml
TEMED	15 µl	15 µl
APS (10%)	80 µl	80 µl
Stacking gel		
Components	Volume (ml) (5% gel)	
Stacking gel 4X buffer	1.25 ml	
Water	3.05 ml	
Acrylamide solution	0.65 ml	
TEMED	7.5 µl	
APS (10%)	40 µl	
Solution for the preparation of the gels		
Rotipherose gel 30	13029 Carl Roth GmbH, Karlsruhe, Germany	
Gel running buffer	25 mM Tris base, 192 mM glycine, 0.1% SDS, adjust pH 8.3	
Resolving gel 4x buffer	1.5 M Tris HCl, 0.4% SDS, pH 8.8	
Stacking gel 4x buffer	0.5 M Tris HCl, 0.4% SDS, pH 6.8	

2.4.16. Silver staining

Fixing solution	40% ethanol, 10% acetic acid, 50% water, 0.05% Formaldehyde (37%)
Silver solution	0.16% silver nitrate, 0.075% Formaldehyde
Developing solution	5% Sodium bicarbonate, 0.025% sodium thiosulphate, 0.04% Formaldehyde (37%)

2.4.17. Western blotting

Protein transfer buffer	3.03 g Trizma-base, 14.4 g glycin, 200 ml methanol, QS aqua bidest
Tris buffered saline (TBS)	12.1 g Trizma-base, 40 g NaCl, diluted to 5 L aqua bidest, pH 7.6
TBS-Tween (TBS-T)	Dilute Tween 20 in TBS to make 0.1% (v/v) solution
PBS	11.5 g Di-sodium hydrogen orthophosphate (anhydrous), 2.96 g Sodium dihydrogen orthophosphate, 5. 84 g NaCl, diluted to 1 L with aqua bidest and pH adjusted to 7.4
PBS-Tween (PBS-T)	Diluted Tween 20n in PBS to make a 0.1% (v/v)solution

2.4.18. ELISA

Blocking solution	PBS, BSA 3%
Coating buffer	0.14 g $NaCO_3$, 0.3 g $NaHCO_3$ (pH 9.6)
PBS	80 g NaCl, 2 g KCl, 11.5 g $NaHPO_4$, 2 g KH_2PO_4
Substrate buffer, OPDA	30 mg OPDA in 30 ml citrate buffer (pH 5.0)
Wash buffer	PBS, 0.05% Tween

2.4.19. Bacterial media and agar

2 YT broth	31 g in 1 L of demineralised water and autoclave for 15 min at 121°C
YT-agar	12 g select agar/L of YT medium and autoclave for 15 min at 121°C

2.4.20. Buffers for protein purification

Lysis buffer	20 mM Tris-HCl pH 8.0
	300 mM NaCl
	10 mM Imidazol
Wash buffer	50 mM Tris–HCl pH 7.6
	1 M NaCl
	20 mM Imidazol
	0.5 % Triton X 114
	50 mM Tris–HCl pH 8.0

Elution buffer	150 mM NaCl 300 mM Imidazol

2.4.21. Kits

Dc Protein Assay	Bio-Rad laboratories, München, Germany
cDNA Synthesis Kit	Fermentas, St. Leon-Rot, Germany
Griess Reagent System	Promega, Mannheim, Germany
Invisorb Plasmid Mini	Invitek (Berlin, Germany)
Jetsorb gel extraction	Genomed, Bad Öyenhausen- Germany
LAL kinetic-QCL	Cambrex Bioscience, USA
LSAB2, DAB	Dako Deutschland GmbH, Hamburg, Germany
PGE_2 monoclonal ELISA	Cayman Chemical Company, Ann Arbour, USA
RNeasy plus-mini;Maxi-plasmid	Qiagen, Hilden, Germany
SensiMix plus SYBR	Quantace Berlin, Germany
SuperScript cDNA Synthesis	Invitrogen, Karlsruhe, Germany
SuperSignal West Pico Chemiluminescent Substrate	Pierce Biotechnology, Jena
Z-Competent *E. coli* transformation buffer set	Zymo Research, USA

2.4.22. Instruments

Amaxa nucleofector	Amaxa Biosystems, Cologne, Germany
BIO-TEK Synergy Microplate Reader/washer	Bio-Tek Instruments, Bad Friedrichshall, Germany
iQ5 real time PCR detection system	Bio-Rad Laboratories, München, Germany
Flow Cytometer	Beckman Coulter EPICS XL-MCL Oakville, Canada
MicrosonTM Ultrasonic cell disruptor XL	Microsinic GmbH. Dortmund
NanoDrop Spectro-photometer ND – 1000	peQLab Biotechnologie, Erlangen, Germany
PCR-Mastercycler Gradient	Eppendorf, Hamburg, Germany

3.0. RESULTS

The present works covers three aspects of *in vitro* studies of inflammatory arthritis. In the first part canine articular chondrocytes were isolated and cultured in 2D and 3D culture systems. Molecular and phenotypic modulations of these cells were monitored using multiple parameters such growth rate, morphology, expression profiles of chondrocyte marker genes including type I collagen, type II collagen, aggrecan, CRTAC1, COMP and Sox9. Attempts were also made to generate a chondrocyte cell line using human telomerase catalytic subunit. The second part of this study focussed on the cloning and expression of canine recombinant cytokines IL-1β and TNFα to develop and characterize an *in vitro* model of inflammatory arthritis using chondrocytes. From canine blood, the said recombinant cytokines were amplified and cloned into bacterial expression vectors to obtain recombinant proteins. These proteins were used as stimulants to trigger an inflammation cascade in chondrocytes in 2D and in 3D cultures. The inflammation was measured by the expression of various inflammatory cytokines, enzyme mediators and their catabolites along with the breakdown products of chondrocyte matrix components particularly type II collagen and aggrecan. The expression of canine IL-4 protein was monitored by Western blot and an ELISA was setup to measure the expressed protein in transduced chondrocytes. Then the anti-inflammatory capacity of IL-4 was observed by its ability to down regulate a number of inflammatory mediatotors in both cell culture systems. This work was extended to see whether the anti-inflammatory action of IL-4 involves STAT6. Since the canine form of STAT6 has not been cloned, STAT6 from this species was partially cloned using primers directed to the predicted isoform from human STAT6 and the products sequenced.

The results of these different experiments are described and documented in detail below.

3.1. Molecular and phenotypic characterization of chondrocytes

Macroscopically normal articular cartilage samples were collected from euthanized dogs for chondrocyte isolation 24 h post-mortem. Average wet weight of the articular cartilage was 3.2 grams and average chondrocyte yield was 3.12×10^6 cells per gram of cartilage. Total cell count was found to be 1×10^7 cells, which were handed usually in suspensions of approximately 1.0×10^6 cells ml^{-1}. Viability of the isolated chondrocytes as measured by trypan blue exclusion dye was always found to be more than 95%. There was apparently no correlation between yield and viability of

chondrocytes with the age of donor or cartilage wet weight for each sample. Approximately, $1x10^6$ cells were seeded in 25 cm^2 tissue culture flasks and cultured in DMEM with 10% FCS and 1% penicillin/streptomycin at 37°C with 5% CO_2. Freshly isolated chondrocytes adhered onto the tissue culture flasks within 24-72 h of culture. The adherence time appeared to be directly related to the age of the dog. Chondrocytes settled as early as 24 h in younger dogs and as late as 72 h in older dogs (compare Table 2. Multiplication of cells started on the second or third day and they became prominent monolayer after 5-6 days of culture.

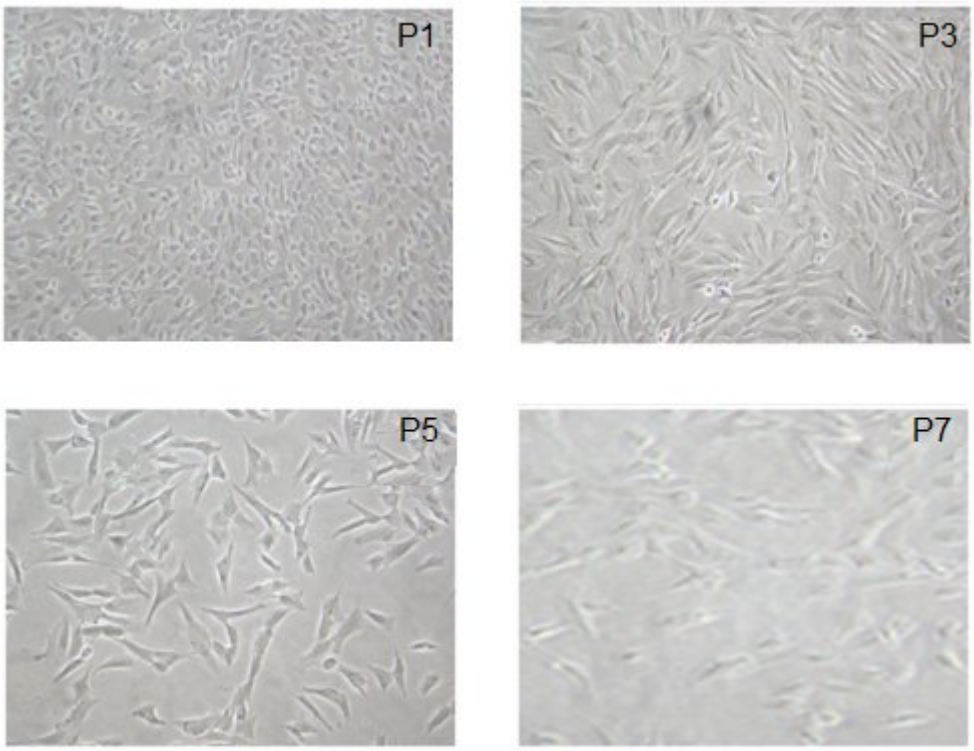

Fig. 6: Microscopic morphology of canine chondrocytes in 2D culture. Canine chondrocytes were isolated from articular cartilage by enzymatic digestion and cultivated in 2D for up to P7. All the passages were maintained in DMEM enriched with 10% FCS and 1% penicillin/streptomycin under standard incubation conditions (37°C, 5% CO_2, 95% humidity). The microscopic phenotype of chondrocytes at selected passages is shown (magnification 200x). The characteristic polygonal morphology of chondrocytes changed to fibroblast-like cells after serial passaging where they appear more elongated or spindle shaped.

They appeared uniform in size with polygonal shape and were distributed on the substratum as islet of growth. Cells in primo culture (P0) reached confluence within 1 week and remained polygonal in shape. As the cultured chondrocytes were serially passaged, they adopted an elongated or fibroblast-like shape. At this stage,

chondrocytes grew more slowly and took longer to reach confluence. After four passages (P4), cultured chondrocytes were more or less spindle like in shape and appeared larger in size when compared to the cells in early passages. Chondrocytes at P7 were very slow in proliferation and only reached a sub-confluent state at the end of 3 weeks of culture and remained unable to achieve confluence (Figure 6).

Viability of the cells at any phase of passaging always exceeded 95%. There was no considerable variation of viability between different culture periods. Cells were propagated up to passage 7 (P7) because they were unable to reach confluence at this stage. These results show that chondrocytes in 2D assumed the characteristic fibroblast-like, flattened and elongated morphology, which has been consistently reported in the literature for articular chondrocytes from several species.

3.1.1. Growth rate and morphological changes

During 2D culture chondrocytes were counted at each passage and average numbers of cells per passage were calculated to determine the growth rate. The average cell number decreased first through P7 in all cultures. Similarly, the growth rate of chondrocytes as determined by a mathematical equation was found to be highest in P0 and then decreased with every following passage in 2D culture. It is therefore evident that cultured chondrocytes achieved the average increase in cell number in the P0- P3 as compared to the passages thereafter. This was further confirmed when the growth rate in P0 and subsequent passages was compared. As the cultured chondrocytes were passaged from P0 to P1, their growth rate decreased gradually up to P7, where growth seized completely. Cell multiplication could then hardly be noted under the inverted light microscope. The subconfluent culture was trypsinized after 5-7 days and total cells were counted. In contrast to adhesion efficiency there was no major correlation between chondrocyte growth rate and the age of the donor in this study and chondrocytes cultured in the P1 to P7 stages showed more than 95% viability for all donors. The Average cell count and the growth rate of chondrocytes during passaging are shown in Figure 7.

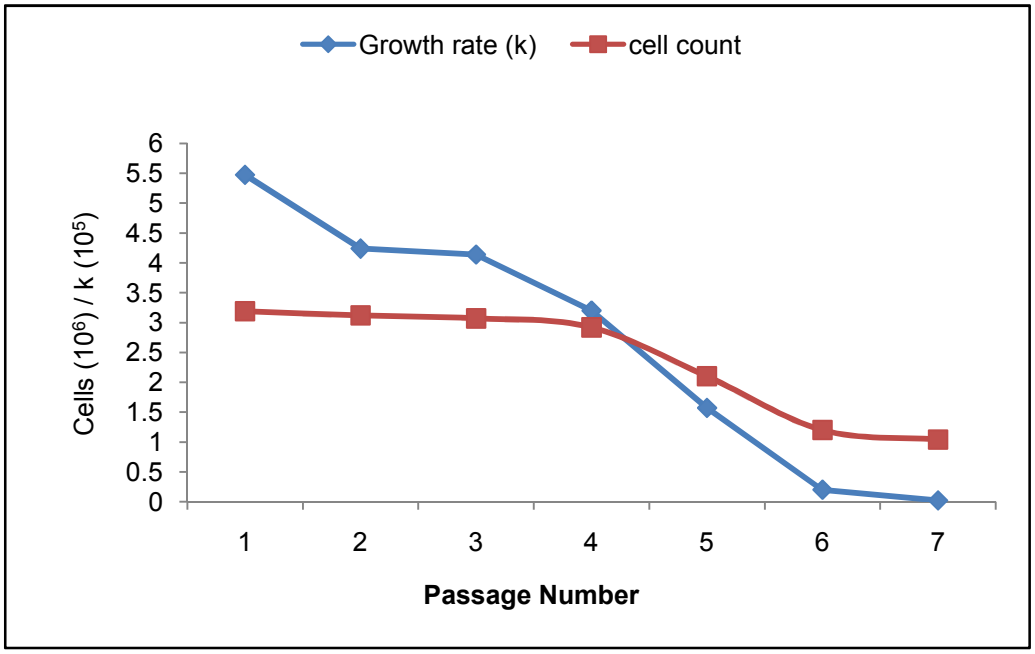

Fig. 7: Average cell count and growth rate of chondrocytes in confluent 2D culture: At each passage (P1-P7) cells were harvested by trypsinization once when they reached confluence. Cells were counted using trypan blue exclusion dye and the average cell number was recorded. The average number of cells decreased with growing passage numbers in 2D culture. Growth constant (*k*) of confluent cultures was calculated using the formula: $k = (\ln N_2 - \ln N_1)/t$. $\ln N_1$ = initial number of cells ($1x10^6$), $\ln N_2$ = number of cells in confluent culture and t = number of days required for reaching confluence. Results show that *k* values decrease by serial passaging and cells in P7 did not reach confluence.

Typically, chondrocytes are polygonal and being primary cells, they usually de-differentiate to fibroblasts during the course of propagation. In this study, chondrocytes showed extensive morphological changes during the cultivation period in 2D. Cells at P0 appeared small in size and spherical to polygonal in shape. In some areas of the tissue culture flaks they formed multilayers. In the late passages they appeared comparatively larger in size, more flattened in shape and had an elongated fibroblast-like phenotype (compare Figure 6). In parallel with these changes, the cell densities of the confluent cultures decreased from passage to passage. Flow cytometry analysis for cell size revealed that populations of cells with subsequent passage moved towards higher values on the forward (FS) axis, which is an indication of enlarging cells during cultivation. Likewise, cells also shifted from a

low to high granularity on the side scatter (SS) axis of the FACScan analysis. Cell size and granularity as determined by flow cytometry are shown in Figure 8.

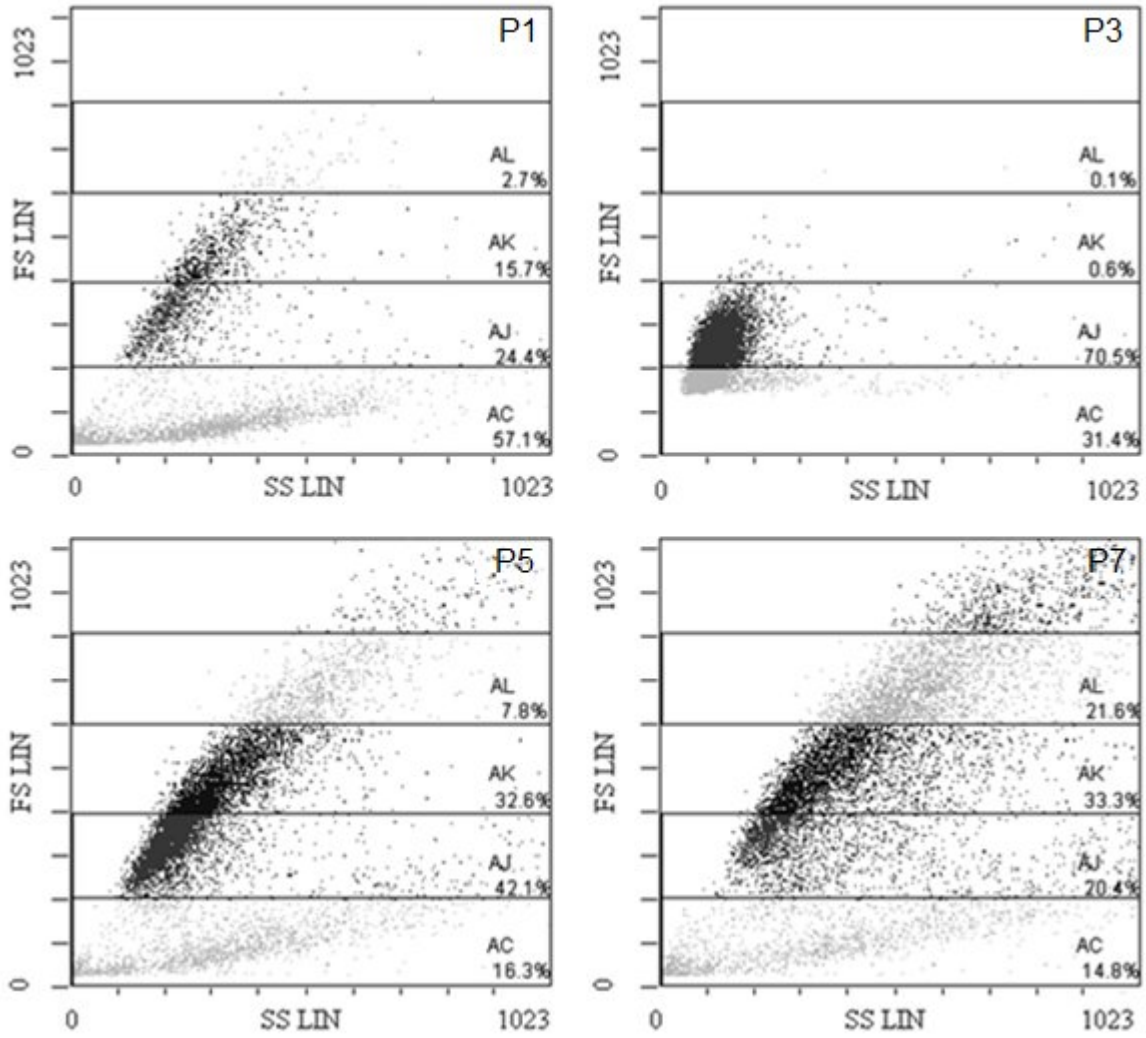

Fig. 8: Size and granularity of chondrocytes in 2D cultures. Chondrocytes were harvested from 2D culture in different passages and subjected to FACScan analysis to monitor their size and granularity. FACScan analysis from first to seventh passage are shown here. Forward scatter (FS) represents cell size which increased along with serial passaging. FS was divided into five different sectors to facilitate the assessment of increase in size of cells by showing the percentage of cells shifted from lower sectors (small cells) to higher sectors (larger cells). Side scatter (SS) shows the granularity which was also increased from P1 through P7. Increase in cell size and granularity signify the change in cell morphology.

3.1.2. Histological and immunohistochemical analysis of cartilage tissue

As described in materials and methods, articular cartilage was stained for collagens as well as proteoglycans. Immunohistochemical staining for collagens was done with Dakocytomation utilizing type I collagen and type II collagen antibodies.

The results (not shown) indicate that in the native cartilage tissue there was high expression of cartilage-specific type II collagen. No expression of type I collagen was

detected in the cartilage sample. Histological Alcian blue staining was done for detection of proteoglycan in the cartilage tissue. This was done to establish the presence of sulfated proteoglycans in the ECM, which was taken as evidence of functional chondrocytes differentiation in native tissue. The results revealed that there was a strong expression of cartilage-specific proteoglycans in the tissue.

3.1.3. Immunocytochemical analysis of chondrocytes in 2D culture

Chondrocytes were cultured in chamber slides and were stained by Dakocytomation using type I collagen and type II collagen specific antibodies for detection of chondrocyte non-specific type I collagen and chondrocyte specific type II collagen respectively. This immunocytochemical staining revealed a low expression of type I collagen at P1 which increased at later passages. On contrary, expression of type II collagen, which was highest at P0 decreased rapidly afterwards. In Figure 9 representative results of immunocytochemical analysis of chondrocytes in various passages are depicted.

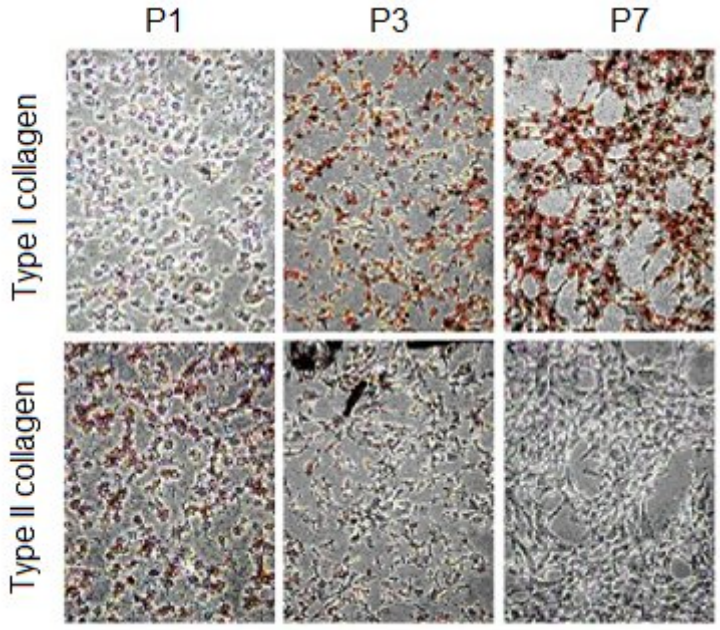

Fig. 9: Immunocytochemistry of chondrocytes for type I and type II collagens. Chondrocytes in different passages (P1, P3 and P7) were cultured in chamber-slides. After fixation cells were incubated with the respective collagen primary antibodies. Detection was made by use of the Dakocytomation technique. It became apparent that with increasing passage number expression of type I collagen increased while that of type II collagen decreased.

The mean Mankin score based on the expression intensity of type I collagen and type II collagen is given in Table 4. Immunocytochemical methods showed that the changes in the patterns of expression of cell-associated collagen types occurred from P3. Expression of intracellular type II collagen was found to be highest in P1 and decreased thereafter with increase in passage number and is virtually absent in P7. Synthesis of type I collagen was apparently detectable in cells in P3 and then its expression was increased for the entire observed cultivation period in 2D culture.

Table 4: Evaluation of immunocytochemical staining

Passage No.	Collagen type I	Collagen type II
P1	+	+++
P3	++	+
P7	++++	-

After immunocytochemical staining, the slides were subjectively evaluated and scored as plus (+) or minus (-) based on the appearance of colour for each collagen type. Representative intensities of expression of type I collagen and/or type II collagen in the 2D culture during P1-P7 are shown. P = passage, + = presence and intensity of expression, - = absent or extremely low expression

3.1.4. Collagen synthesis: Collagen typing with Western blot

The presence or absence of both collagen types in canine chondrocyte was also determined by Western Blot as described under material and methods. The investigation of the synthesized collagen phenotype of chondrocytes grown for different passage number in 2D culture with collagen-specific antibodies showed synthesis of type II collagen only in the P1 which was no more detectable at protein level in successive passages. Detection with collagen type I-specific antibody indicated the beginning of type I collagen synthesis in P2 which increased with an increase in passage number and was present throughout the culture period. Western blot results of both collagen types are shown in Figure 10. Unlike, type II collagen where only one single specific band was seen, type II collagen (141 kDa) showed two bands on the film. Although the intensity of both bands for this collagen type was the same for each passage, the calculated molecular weight for these bands suggest that the lower band corresponds to the molecular weight of type I collagen which should be 137 kDa. It is not known at present what the origin and nature of the upper band may be.

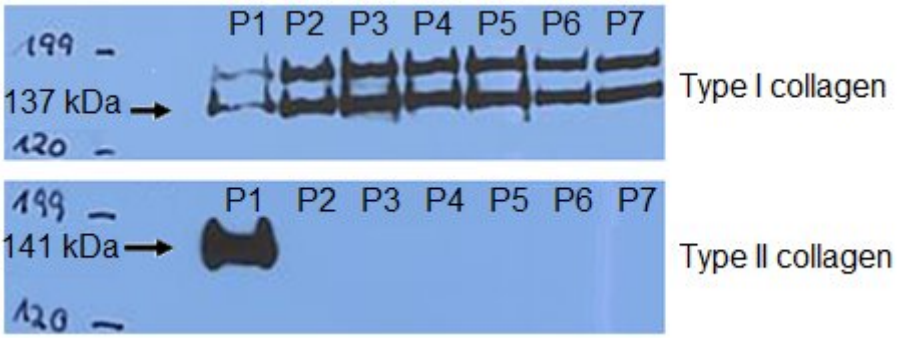

Fig. 10: Western blot analysis of type I collagen and type II collagen proteins. Chondrocytes were harvested from 2D culture. The cells were lysed by RIPA buffer and proteins were loaded on 7% SDS gels. The proteins were electroblotted on PVDF membranes and then incubated with either type I or type II collagens antibodies and detection was made by HRPO conjugate secondary antibodies. Results showed an increase in type I collagen expression with increase in passage number, however type II collagen was detected only in P1.

3.1.5. RNA extraction, cDNA synthesis and RT-PCR and qRT-PCR

For the analysis of chondrocyte marker gene mRNA, cells in 2D (P1-P7) and 3D (1-3 weeks) cultures were harvested and centrifuged at low speed. Sediments were obtained which were then subjected to RNA isolation as described above. Total RNA was reverse transcribed to synthesize first strand cDNA. RT-PCR was applied to amplify the mRNA coding for canine type I collagen, type II collagen, aggrecan, CRTAC1, COMP, and Sox9. GAPDH, β-actin and 18s rRNA were used as housekeeping genes. It was found that GAPDH rather than β-actin and 18s rRNA showed a more stable and consistent expression (results not shown) and therefore GAPDH was selected as the house keeping gene for normalization.

Freshly isolated articular chondrocytes have been shown to produce a matrix of collagens and proteoglycans along with specific markers characteristic of cartilage. In order to assess the changes in the expression patterns and degree of de-differentiation of chondrocytes, quantification of mRNA expression was performed by qRT-PCR. The expression of above mentioned genes measured in order to follow the gradual loss of these differentiation markers and the steady-state level of mRNA prior to alginate inclusion. The RT-PCR analyses (not shown) confirmed that, at the time of isolation P0 the normal canine articular chondrocytes were fully differentiated, expressing type II collagen, aggrecan, CRTAC1, COMP and Sox9 mRNAs but not type I collagen. The cells kept these differentiated characteristics for the few early

passages and then the expression was drastically switched from cartilage specific molecules to cartilage non-specific molecules. As seen from the qRT-PCR results in Figure 11 the mRNA expression of type I collagen remained almost undetectable until P3 after which it was extensively expressed, and its expression continued to increase until P7. This type of collagen was the predominant form of collagen in the later passages. Type II collagen mRNA was expressed strongly in freshly isolated cells but had become barely detectable in confluent cells at or after P3. With regard to the reference gene, GAPDH, the normalized expression of type II collagen for the entire 2D culture decreased several fold compared with that at the beginning of culture. The mRNA expression of other chondrocyte marker genes such as aggrecan, CRTAC1, COMP and Sox9 followed similar kinetics and the level of their expression dropped dramatically at or after P3 and was barely detectable in later passages indicating de-differentiation of chondrocytes.

Gene expression of chondrocytes in alginate beads was also investigated using the above mentioned chondrocyte markers. The expression of type I collagen was decreased down to levels below detection with the time of cultivation in alginate beads. The expression of the remaining marker genes except CRTAC1 was still very low 7 days after the inclusion in alginate, but had clearly resumed by day 14 and kept increasing during the 3rd week of encapsulation in alginate microspheres.

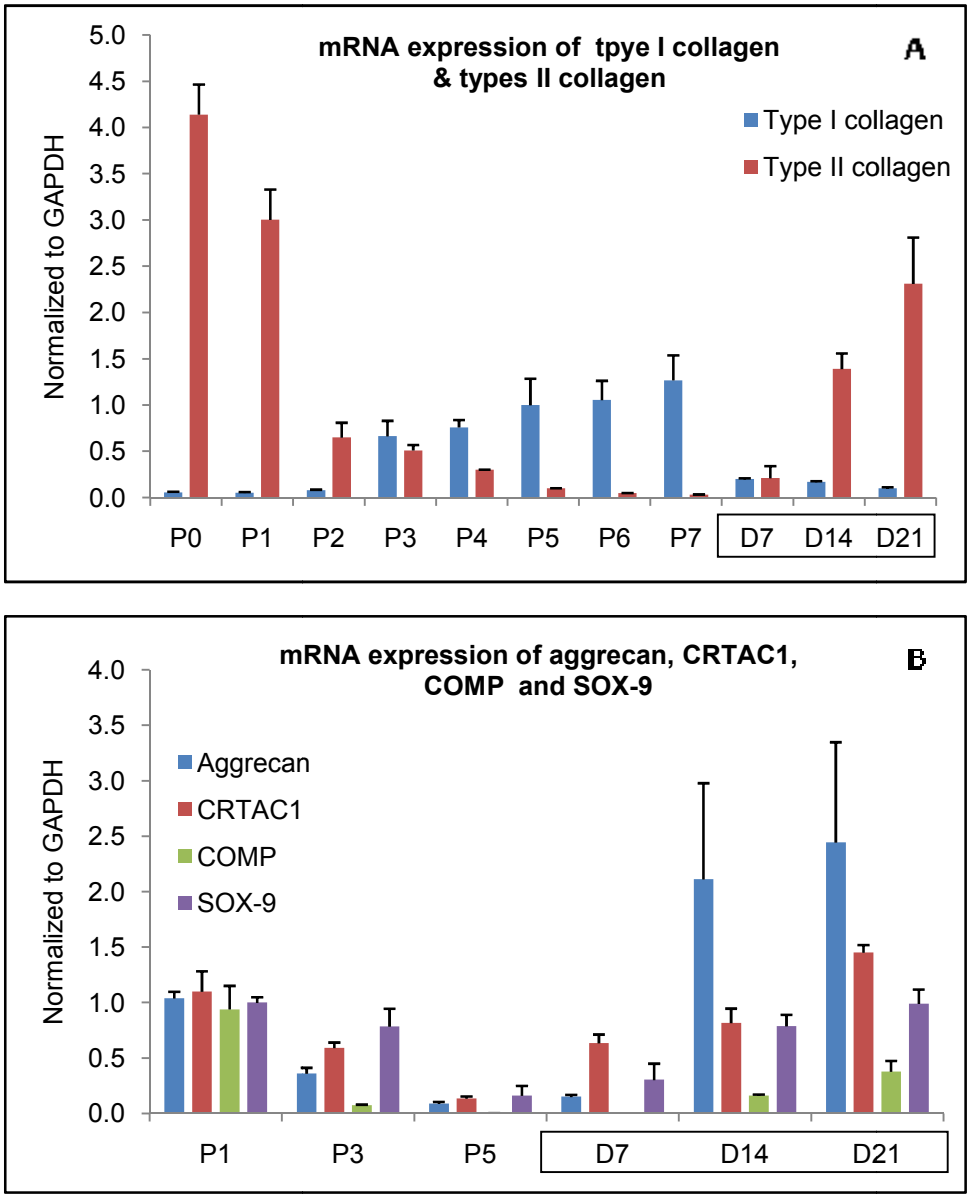

Fig. 11: Expression of various chondrocyte marker genes. Chondrocytes after isolation from canine cartilage were cultured in 2D for up to P7 and in 3D culture for 21 days. Total RNA was extracted from chondrocyte pellets and was reverse transcribed to synthesize cDNA. mRNA expression was quantified by qRT-PCR for various chondrocyte marker genes relative to the house keeping gene (GAPDH). It is apparent that type I collagen expression increases with growing passage number and decreases again when subjected to 3D culture. The reverse is true when expression of type II collagen is assessed. (A). The expression of aggrecan, CRTAC1, COMP and Sox9 in different passages and after 7, 14 and 21 days in 3D culture was decreased with subsequent passage, but increased again when the cells were subjected to alginate microspheres to form 3D cultures (B).

3.1.6. Differential index

The type II collagen/type I collagen ratio (CII/I) defined as a ‘’differentiation index’’ decreased during cultivation in 2D and increased in alginate microspheres (Figure 12). The differential index was calculated using end point values from the qRT-PCR data as generated by the software. The index was higher in the early passages and decreased with time when cells were serially passaged in 2D cultures. However, after the cells were encapsulated in alginate beads this ratio began to rise from 1-3 weeks of 3D culture.

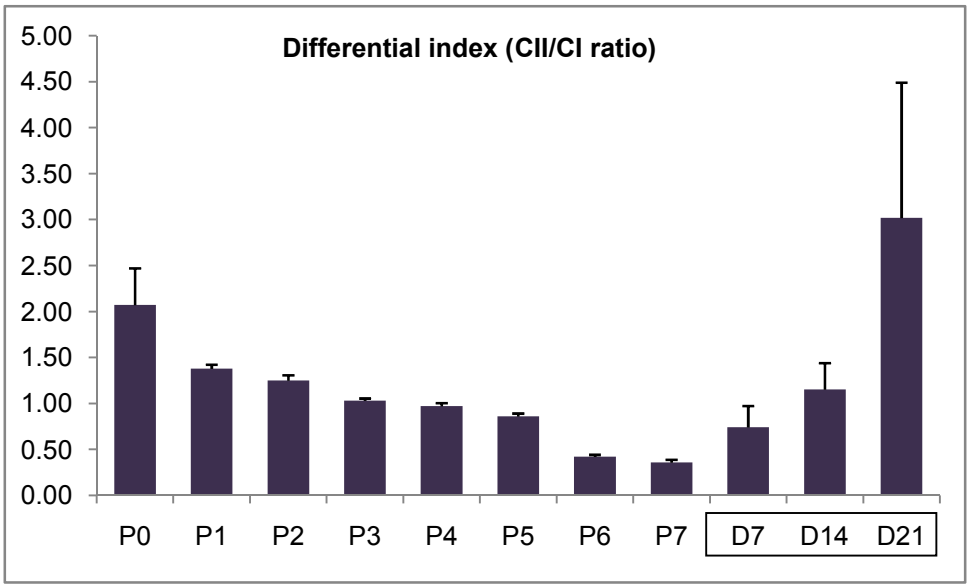

Fig. 12: Differentiation index in 2D and 3D cultures. Gene expression for type I collagen and type II collagen was quantified by qRT-PCR and the ratio between cartilage specific type II collagen and cartilage non-specific type I collagen was calculated.

To summarize, chondrocytes cultivated in 2D rapidly divide and lose their morphological and biochemical characteristics, whereas they regain and maintain their phenotype for long periods of time when they are cultivated in alginate beads. Because cartilage has a low cellularity and is difficult to obtain in large quantities, the number of available cells often becomes a limiting factor in studies of chondrocyte biology. Therefore, the possibility of restoring the differentiated properties of chondrocytes by cultivating them in alginate beads after two multiplication passages in 2D was explored. This resulted in the re-expression of all the main markers of differentiated chondrocytes: type II, Collagen aggrecan, CRTAC1, COMP and Sox9 gene expression was strongly re-induced from day 7 after alginate inclusion. However, three weeks of 3D culture were necessary for total suppression of type I collagen synthesis which is an indicator of the modulated phenotype.

3.1.7. Assessment of transfection efficiency in chondrocytes

Transfection efficiencies are controlled by a variety of parameters. Two different transfection techniques FuGENE 6 and Amaxa nucleofection were tested to observe the optimal transfection efficiency. We used the optimized protocols for both techniques with equal amounts of plasmid DNA for each transfection reaction and chondrocytes in the same passage served as target cells. FuGENE 6 method was optimized by testing various FuGENE 6:DNA ratios while the Amaxa method was optimized by selecting different transfection cocktails and nucleofection programs. Transfection efficiency was assessed by FACScan analysis after expression of the green fluorescence protein. The transfection technique strongly influenced the yields of transfection. Non-liposomal FuGENE 6 yielded a transfection efficiency of 51.7% compared to the electroporation nucleofector method which resulted in 68.1% transfection efficiency. However, although the Amaxa nucleofection yielded higher transfection efficiency, the number of dead cells remained a constant problem. Also, the nucleofected cells require more time to settle after transfer to the culture flasks. Therefore, Amaxa was generally preferred for stable transfection. FuGENE 6 was routinely used for transient expression where immediate supply and more viable cells were warranted.

3.1.8. Generation of chondrocyte cell line

In order to increase the life span of the chondrocytes, cells were transfected with hTERT. Cells were grown continuously over a period of several weeks (up to P20) to determine the effects of hTERT on growth characteristics and phenotype. Expression profiles of type I collagen and type II collagen and other typical chondrocyte markers were determined as described in materials and methods.

The plasmid DNA was freshly transformed and restriction digestion with EcoRI and SalI confirmed that the insert size of 3405 bp was released. The sequencing results showed 100% homology with the published sequence of human telomerase catalytic subunit, mRNA (Accession No. AF018167).

3.1.9. Phenotype and growth rate

The pCIneo-hTERT transfected as well as mock-transfected control cells were grown with G418 for 2 weeks. The non-transfected cells started to die within 2 days of G418 treatment. After 1 week there was heavy loss of non-transfected cells and approximately 90% cells detached from the culture dishes and started to float in the

medium and on day 10 no viable cells were detected in the culture plate. In transfected cells hardly any death was observed in the first week of culture, however after day 10 of incubation approximately 60-70% cells died and after 2 further weeks the death rate remained constant. The G418 dose was reduced to 20% of the original concentration and the cells were always maintained under this lower concentration of the selection antibiotic. Now the cells started to form small colonies at different parts of the tissue culture plates and appeared round to polygonal in shape. Once the colonies had become larger, cells were trypsinized, counted and seeded into a 96-well plate at 1 cell per well. After 2 weeks individual colonies started appearing in the plate which were first cultured individually and later on pooled together to study various parameters. The cells appeared round to polygonal in shape even after P20 (Figure 13).

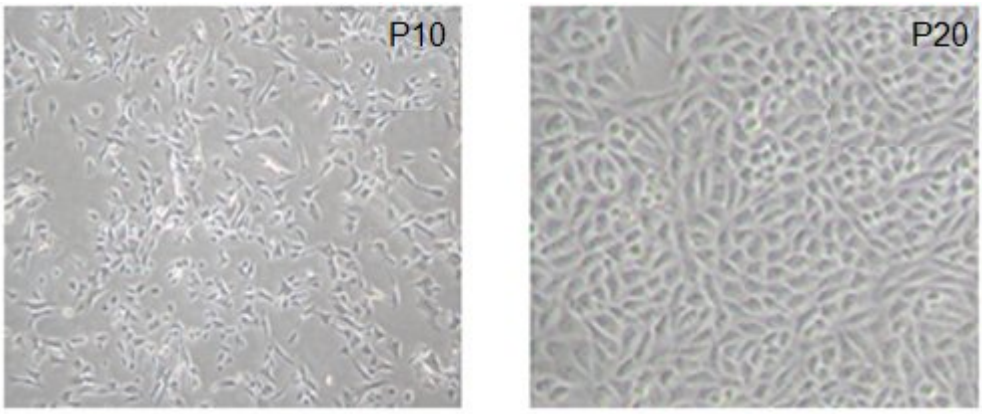

Fig. 13: Microscopic morphology of chondrocyte cell line. Canine chondrocyte cell line (GD-hTERT) in P10 and P20 are shown for light microscopic morphology (magnification 200X). The canine chondrocytes were transfected with hTERT, selected and maintained by G418 sulphate to preserve chondrocyte morphology. There were no remarkable changes observed in size and shape of these cells even after P20 in 2D. Results from representative samples are shown here.

The cell number and growth rate were calculated for each passage until P20. In spite of fluctuations in the growth rate, these cells maintained a round to polygonal morphology unlike primary chondrocytes in later passages. The growth rate was much higher than for primary chondrocytes. Cell number and growth rates at the different passage number of these “immortalized” chondrocytes are shown in Figure 14.

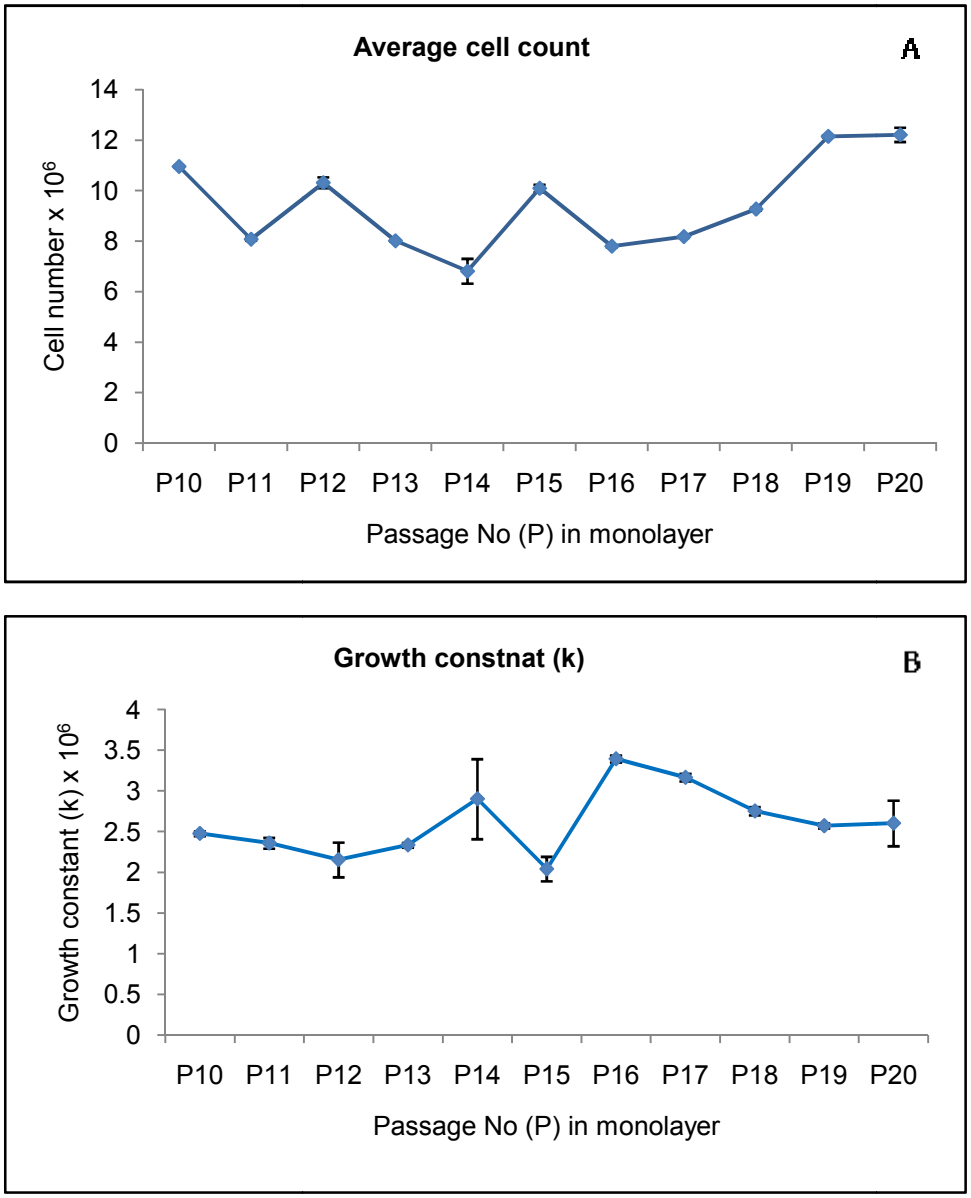

Fig. 14: Cell count and growth rate of chondrocyte cell line (GD-hTERT) in confluent cultures: At each passage, when the cells reached confluence, they were trypsinized and counted using trypan blue exclusion dye. The average number of cells did not change dramatically between passages. Values represent the mean from three independent assessments (A). Growth constant (*k*) of confluent cultures during 2D was calculated by the formula: $k = (\ln N_2 - \ln N_1)/t$. N_1 = initial number of cells ($1x10^6$), N_2 = the number of cells in confluent cultures (N_2) and t = the number of days required for reaching confluence (t). The growth rate did not drop and was flexible during cultivation (B).

3.1.10. mRNA expression of multiple chondrocyte marker genes

Since expression analyses by RT-PCR showed that the “immortalized” cells did not express type II collagen in 2D cultures, they were subjected to a 3D culture system as described for normal chondrocytes. By this procedure they were apparently

stimulated to synthesize chondrocyte specific marker genes like type II collage, aggrecan, CRTAC1, COMP and Sox9. In parallel to this, the highly expressed type I collagen in 2D decreased in the 3D cultures of GD-hTERT cells which indicates re-differentiation of the transformed chondrocytes (Figure 15).

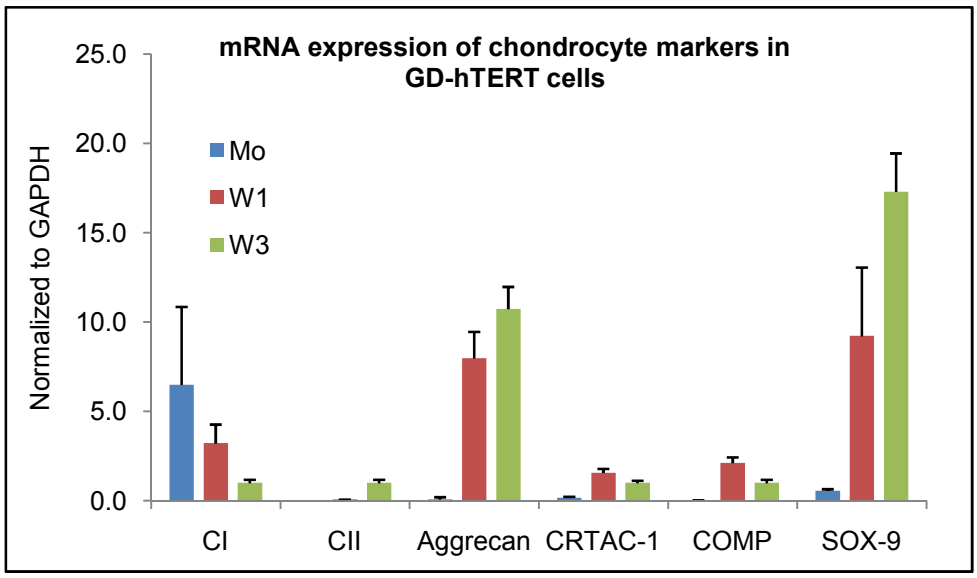

Fig. 15: Expression of various chondrocyte marker genes in GD-hTERT cells. qRT-PCR showing quantitative mRNA expression of type I collagen (CI), type II collagen (CII), aggrecan, CRTAC1. COMP and Sox9 normalized to GAPDH expression in different chondrocyte cell line GD-hTERT in 2D (Mo) and after 1st and 3rd week (W) in 3D culture. The expression of various marker genes for chondrocytes is regained (except CI) when cells were subjected to alginate microspheres.

3.2. Characterization of 3D model of inflammatory arthritis

3.2.1. Molecular cloning and sequence analysis

In order to cause “inflammation” cytokines are required. Since canine cytokines were not available at the time, the required genes were opted to clone and express from canine RNA. A schematic overview of molecular cloning of IL-1β and TNFα is shown in Figure 16. Full length cDNA of canine IL-1β and TNFα were amplified from canine PBMC as described in materials and methods. The fragments shown in Figure 17, corresponded to 798 bp and 713 bp respectively for canine IL-1β and TNFα which are in agreement with the previously published sequences of canine forms. These cytokines were expressed only in PBMC that were stimulated with the respective human cytokine proteins for 72 h under cell culture conditions. In contrast, cDNA from non-stimulated cells did not show any amplification. These amplicons were further sequenced and found to be homologous with those of the published sequences. The sequencing data showed respectively a 99% and 100% homology of these clones to the published canine sequence at the nucleotide level.

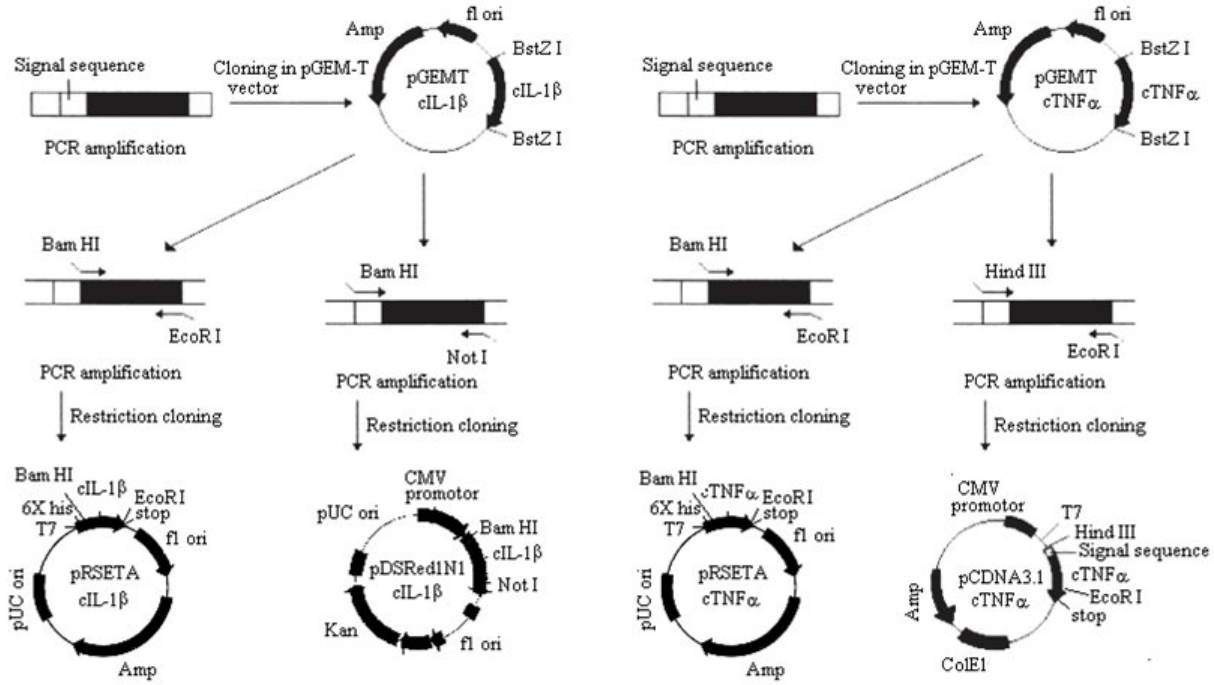

Fig. 16: Construction scheme of cloning and expression vectors for canine IL-1β/TNFα. Canine PBMC were stimulated with human recombinant IL-1β/TNFα. RNA was extracted for RT-PCR and amplicons were cloned into a pGEM-T vector. From this clone, two expression clones were derived and only the coding region of the mature protein was used for bacterial expression system.

Subsequently, primers were designed for amplification of respective proteins without their signal peptide sequences in order to be expressed in the *E. coli* system. Signal

peptide regions were determined based on their hydrophobicity and also cross-checked with the published sequences from the other species. Respective regions for both IL-1β and TNFα upon amplification gave 444 and 455 bp.

The nucleotide sequence homology of IL-1β and TNFα with different species is shown in Table 5. The nucleotides of these clones were translated into amino acids and sequence homology was assessed with published canine, human, mouse and bovine counterparts (Table 6).

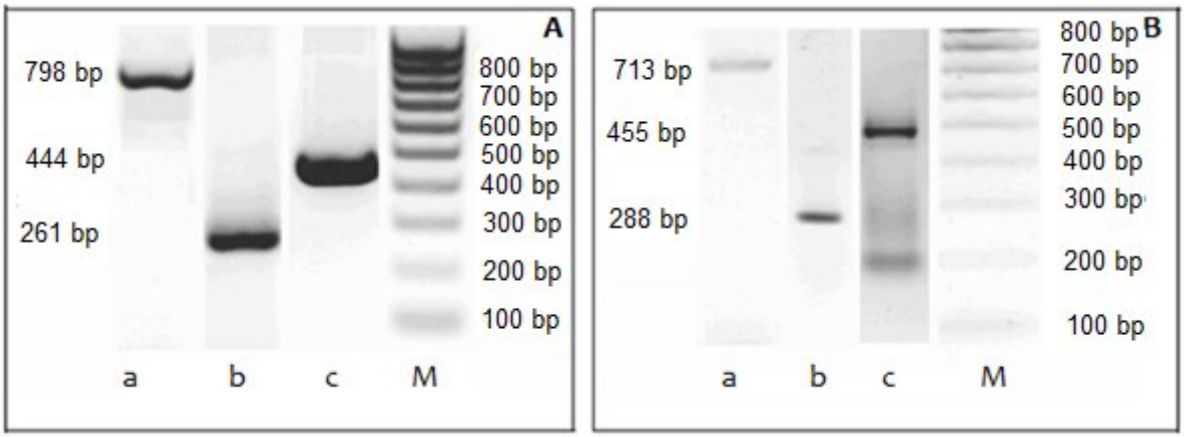

Fig. 17: Reverse transcription PCR for IL-1β and TNFα. Canine PBMC were isolated by Ficoll-Paque Plus and stimulated for 72 h with human recombinant IL-1β (100 ng/ml). Total RNA was extracted and reverse transcribed into cDNA. RT-PCR was used to amplify cDNA coding to full-length (a, 798 bp), internal (b, 261 bp) and mature protein fragments (c, 444 bp) of recombinant IL-1β (A) and to full-length (a, 713 bp), internal (b, 288 bp) and mature protein fragments (c, 455 bp) of recombinant TNFα (B). bp = base pairs (nucleotides), M = DNA ladder

Table 5: Nucleotide homology of IL-1β and TNFα with published sequence from different species

Species	IL-1β (Accession No. EU249360)			TNFα (Accession No. EU249361)		
	Accession No.	Nucle-otides	Homology (%)	Accession No.	Nucleo-tides	Homology (%)
Canine	DQ251036	798	99	NM_001003244	855	100
Human	NM_000576	810	78	X02910	702	91
Mouse	NM_008361	810	74	X02611	708	81
Bovine	NM_174093	801	77	NM_173966	705	83

Table 6: Protein homology of IL-1β and TNFα with published sequence from different species

Species	IL-1β (Accession No. ABX26050)			TNFα (Accession No. ABX26051)		
	Protein I.D.	Amino acids	Homology (%)	Protein I.D.	Amino acids	Homology (%)
Canine	ABB73214	265	99	NP_001003244	233	100
Human	NP_000567	269	54	CAA26669	233	90
Mouse	NP_032387	269	59	CAA26457	235	80
Bovine	NP_776518	266	62	NP_776391	234	79

3.2.2. Prokaryotic expression of recombinant canine IL-1β and TNFα

The respective gene sequences without their signal peptides were cloned into pRSET-A vector that expresses inserted gene sequences under T7-RNA polymerase as His-tag fusion proteins. The expression studies were carried out in a BL21(DE3)p(Lys) host using 1 mM IPTG as mentioned in materials and methods. Both the recombinant proteins were found to be predominantly expressed in the cytosolic fraction, which allowed processing under native conditions. Subsequently, the recombinant proteins were purified from bacterial extracts using Ni-NTA agarose affinity chromatography. Analysis by SDS-PAGE confirmed the over-expressed protein band as approximately 20 kDa which is in agreement with the calculated molecular weight of both the genes. Silver staining was performed to exclude the presence of impurities that would have co-purified (Figure 18A-B).

In the absence of commercially available antibodies for the respective recombinant proteins, anti-His antibody was used for Western blot analysis as this shows the expression of both IL-1β and TNFα fusion proteins. Figure 18C shows the Western blot results from all eluates collected during affinity chromatography. *Bona fide* canine IL-4 was run as a positive control (lane +) which denoted a His-tag protein of size 13 kDa.

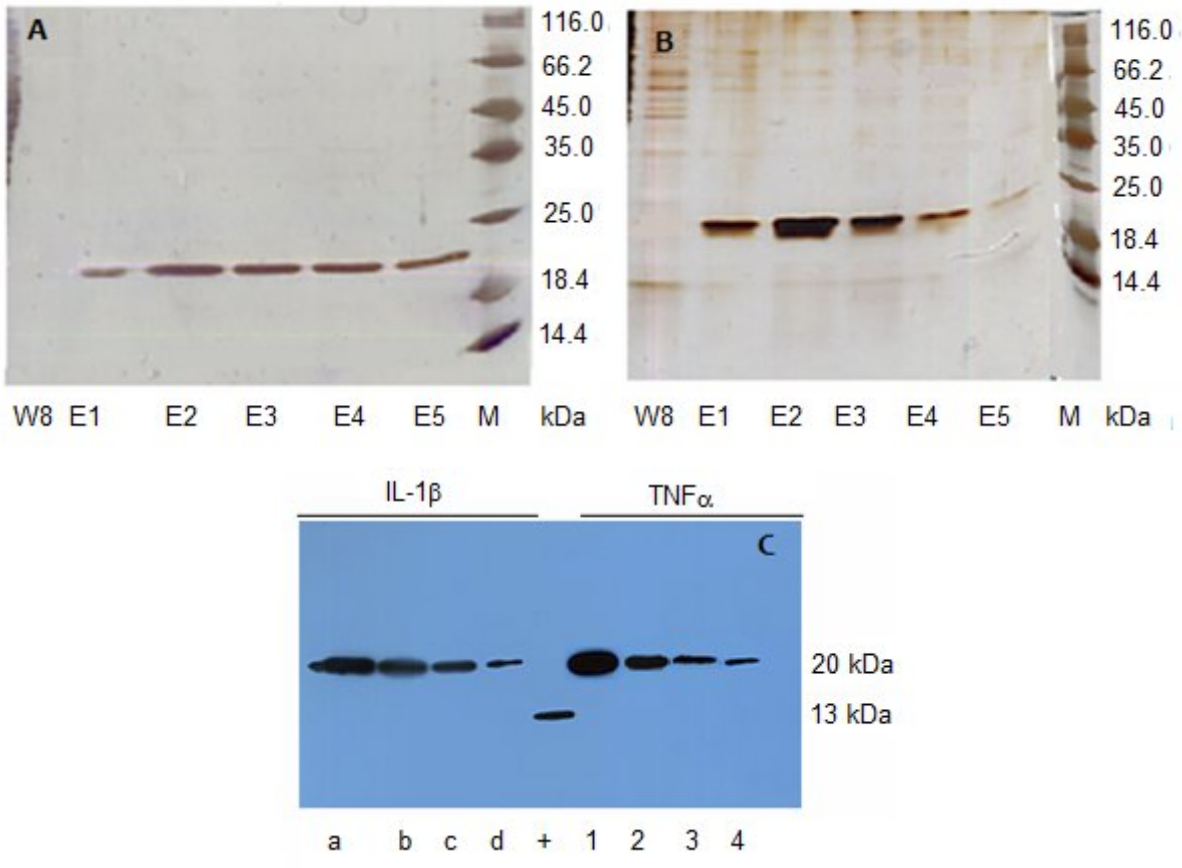

Fig. 18: *E. coli* expression of recombinant IL-1β and TNFα protein. cDNA encoding mature protein region was cloned in bacterial expression vector pRSET-A and transformed in BL21(DE3)p(Lys) strain of *E. coli*. Positive clones in bacterial culture medium were stimulated with IPTG and recombinant proteins were purified using Ni-NTA chromatography. Last wash (W8) and all eluates (E1-E5) after purification were collected, run on 15% SDS gels and stained by Coomassie blue (not shown) and silver. Silver stain as shown IL-1β (A) and TNFα (B) identified each protein of approximately 20 kDa in size. All eluates from the respective protein were pooled and their concentrations measured. For both recombinant proteins, respectively, 200 ng, 100 ng, 50 ng and 25 ng were run on a 15% SDS-PAGE. The proteins were electroblotted and detection was made using anti-his antibody conjugated with HRPO. Western blot results as shown for IL-1β (left) and TNFα (right) identified each protein of approximately 20 kDa in size. As a positive control (+) canine IL-4 protein (13 kDa; previously cloned in this lab) was used at 50 ng concentration (C). M = protein marker, a, 1 = 200 ng, b, 2 = 100 ng, c,3 = 50 ng, d,4 = 25 ng, kDa = kilo Dalton.

3.2.3. Effects on pro-inflammatory cytokines and enzyme mediators

While expression of the respective proteins has been verified by SDS gel electrophoresis and by Western blot analysis (see above), the functional properties of the two recombinant proteins was ascertained by using them to stimulate canine articular chondrocytes.

Chondrocytes in 2D and 3D cell culture systems were established and the effects of recombinant proteins on chondrocytes tested after adding 100 ng ml^{-1} of IL-1β and 50 ng ml^{-1} of TNFα either alone or together to the cell culture medium. Total RNA was extracted from stimulated and non-stimulated cells and assayed for secretion of inflammation related mRNAs. We found that recombinant IL-1β and TNFα induced the expression of pro-inflammatory cytokines, IL-1β, IL-6, IL-8, TNFα and GM-CSF. The transcription levels were quantified by qRT-PCR in 2D and 3D culture systems yielding the results shown in Figure 19A and B, respectively. It becomes apparent that in 2D cultures the levels of most of the inflammatory cytokines are increased with the growing time period of stimulation. In 3D culture, expression of all cytokines mentioned above peaked at 24 h and decreased again at 48 h or 72h of stimulation. In both 2D and 3D cell culture systems, non-stimulated cells expressed hardly any inflammatory marker genes.

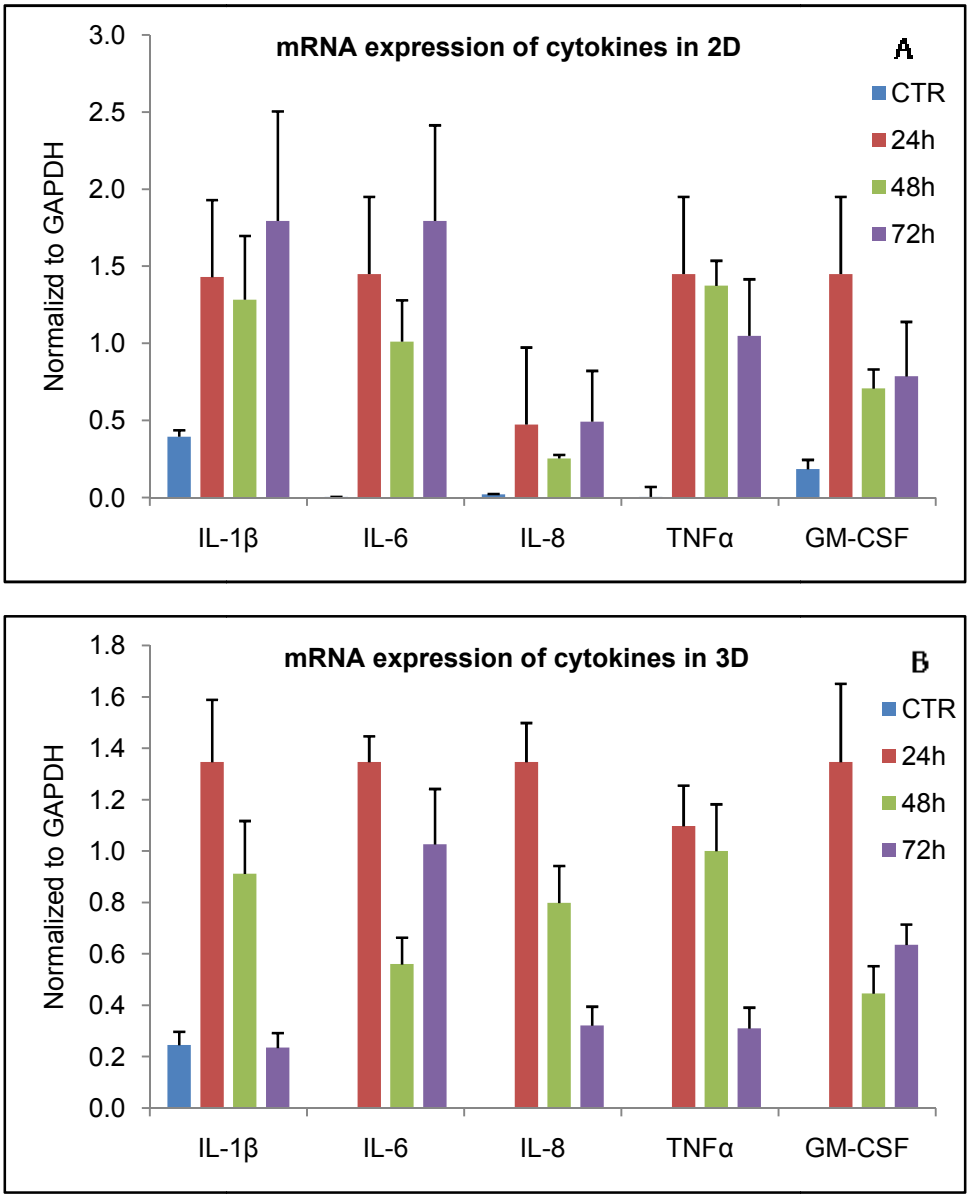

Fig. 19: mRNA expression of inflammatory cytokines in 2D and 3D cultures. Chondrocytes isolated from canine cartilage were stimulated with recombinant canine IL-1β (100 ng/ml) and TNFα (50 ng/ml) for 24 h, 48 h and 72 h in 2D (A) and 3D cultures (B). Total RNA was extracted from the chondrocyte pellet and was reverse transcribed to synthesize cDNA. mRNA expression was quantified by qRT-PCR for IL-1β, IL-6, IL-8, TNFα and GM-CSF relative to the house keeping gene (GAPDH). High expression of inflammatory cytokines was observed in stimulated cells only when as compared to non-stimulated control cells (CTR).

Similarly, iNOS, COX-2 and associated NO and PGE_2 expression were observed only in stimulated cells. However, iNOS expression was found to be higher at 24 h than after 48 and 72 h of stimulation (Figure 20A). Because iNOS leads to the production of NO, the catabolites of NO *viz.,* nitrites were quantified calorimetrically

by the Griess reagent as mentioned in materials and methods. Contrary to the transcript levels of iNOS, it appeared that nitrites accumulated over time and hence showed a steady increase of levels over the full time span of nitrites both in 2D and 3D cell culture systems (Figure 21A).

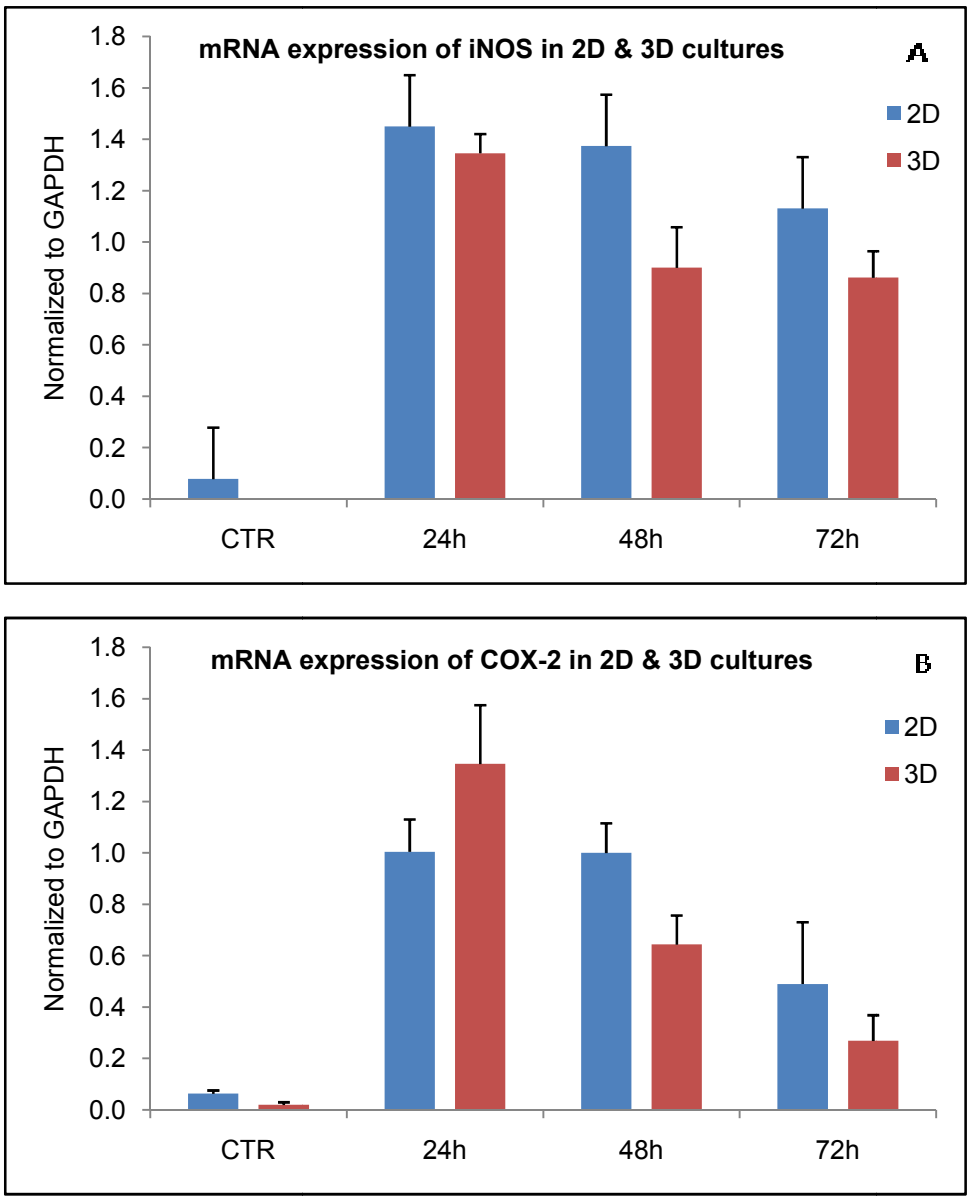

Fig. 20: mRNA expression of iNOS and COX-2. Chondrocytes isolated from canine cartilage were stimulated with recombinant IL-1β (100 ng ml^{-1}) and TNFα (50 ng ml^{-1}) for 24 h, 48 h and 72 h in 2D and 3D cultures. Total RNA was extracted from chondrocyte pellet and reverse transcribed to synthesize cDNA. mRNA expression was quantified by qRT-PCR relative to the house keeping gene (GAPDH). A high expression of iNOS (A) and COX-2 (B) was observed in stimulated cells as compared to non-stimulated control cells (CTR).

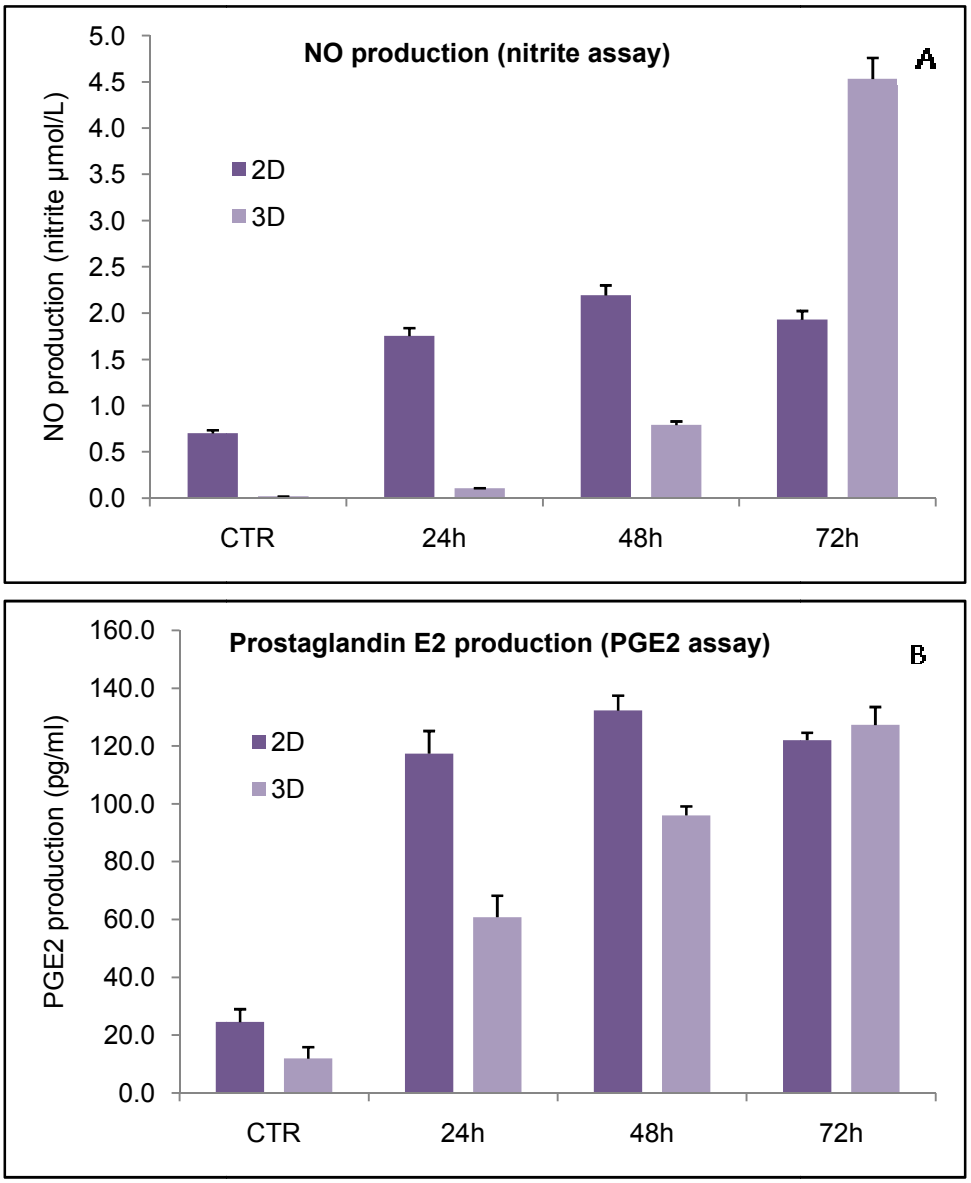

Fig. 21: Nitrite and Prostaglandin E_2 determination. Chondrocytes from canine cartilage were stimulated with recombinant IL-1β (100 ng ml^{-1}) and TNFα (50 ng ml^{-1}) for 24 h, 48 h and 72 h in 2D and 3D cultures. Supernatants were collected from stimulated and non-stimulated cells. NO production was determined by measuring nitrite in the culture medium assay based on the Griess reagent system. Concentration of nitrite is plotted (A). PGE_2 was assayed by Cayman's PGE_2 EIA kit. The amount of PGE_2 produced is expressed as concentration in pg/ml. (B).

The other important mediators of inflammation namely COX-2 and PGE_2 were also recorded both at mRNA level and calorimetrically respectively. Similar to iNOS, COX-2 also showed high expression levels at 24 h which seized after prolonged stimulation (Figure 20B). Similar to nitrite levels, PGE_2 concentrations also increased at 48 and 72 h of stimulation in the 3D culture system. In contrast, the assessed 2D

cell culture system showed similar levels at all time periods of stimulation (Figure 21B).

3.2.4. Effect on MMPs, aggrecan and type II collagen expression

While the inflammatory cytokines and other mediators represent the progression of inflammation, the other side of arthritis is represented by cartilage degradation. This was estimated by assessing the presence of proteolytic enzymes, namely MMPs which were expected to be stimulated by IL-1β and TNFα.

As seen from Figure 22A, canine recombinant IL-1β and TNFα produced these MMPs which in turn showed an effect on structural proteins of chondrocytes *viz.,* collagen II and aggrecan (see Figure 22B). It is apparent that IL-1β and TNFα could increase MMPs and that their expression profile was consistent with other inflammatory makers (compare Figure 19). Accordingly, type II collagen and aggrecan levels suffered drastically under the influence of the recombinant cytokines. Their expression profile declined smoothly with increase in stimulation time (Figure 22B).

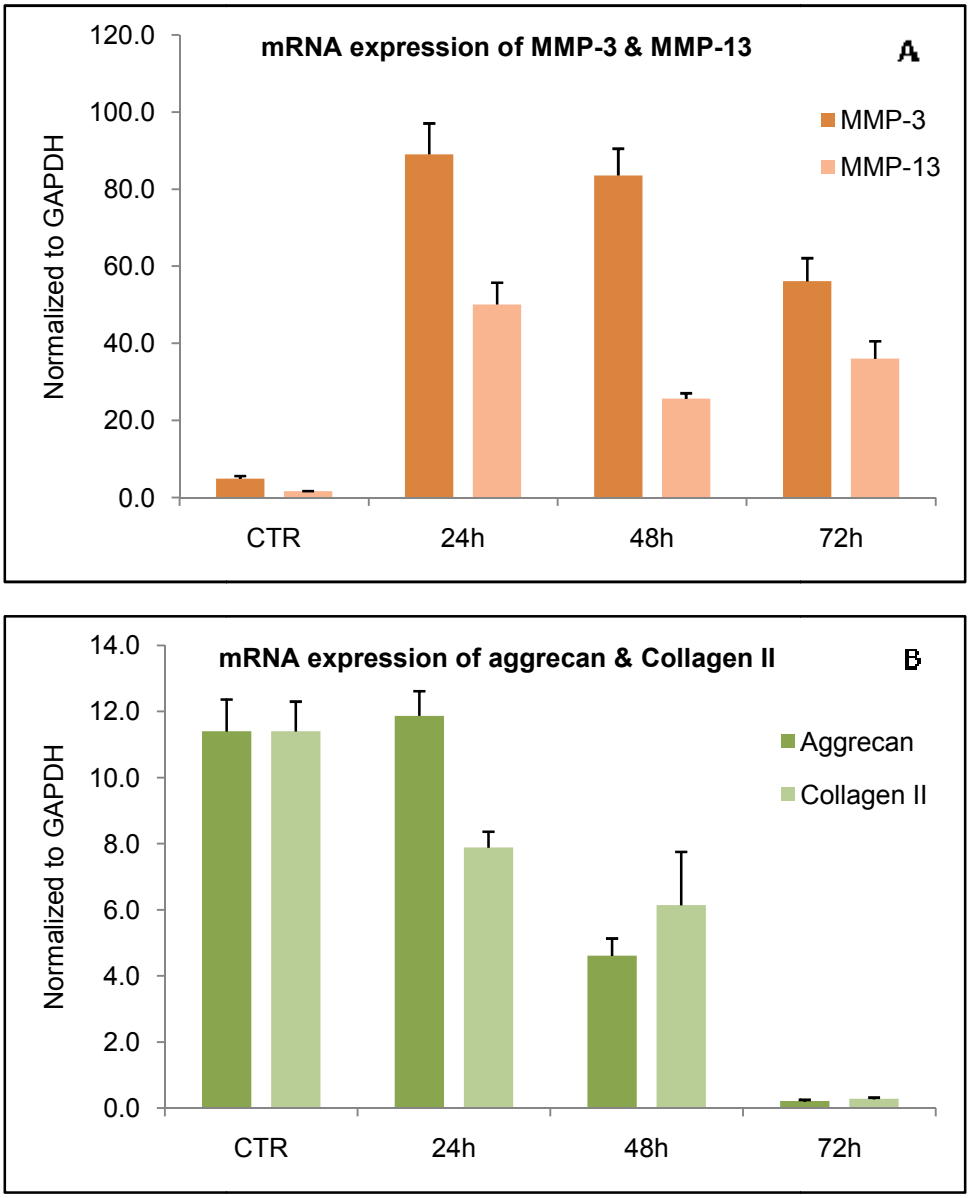

Fig. 22: mRNA expression of MMPs, and aggrecan and type II collagen. Chondrocytes isolated from canine cartilage were stimulated with recombinant IL-1β (100 ng/ml) and TNFα (50 ng/ml) for 24 h, 48 h and 72 h in 2D culture. Total RNA was extracted from the chondrocyte pellet and reverse transcribed to synthesize cDNA. mRNA expression was quantified by qRT-PCR relative to the house keeping gene (GAPDH). A high expression of MMPs (A) and a decreased expression of both aggrecan and type II collagen (B) was observed in stimulated cells. Non-stimulated cells were analyzed as control (CTR).

3.3. IL-4 transgene expression in chondrocytes

Transient gene expression is often used as a preliminary test of gene expression. The production of a protein from transfected cells is usually assayed from the cell culture after a short period, usually 48-72 h following the uptake of DNA. We used a transient expression system involving primary chondrocytes with a mutant SV40 virus having an inactivated origin of DNA replication. Thus any transfected DNA containing SV40 origin of replication can be replicated in these cells. The pCDNA3.1-IL-4 plasmid, which was previously constructed, was transfected in primary chondrocytes using FuGENE 6 or Amaxa nucleofector. The expression and secretion of this protein from chondrocytes was detected 72 h after transfection culture and expression was measured by Western blot analysis and ELISA. Different monoclonal antibodies which have been previously raised against canine IL-4 were also tested and it was found that monoclonal antibody mp41 has highest efficiency as it was able to detect recombinant IL-4 protein (results not shown). Based on these results, mp41 antibody was used as a secondary detection antibody in ELISA.

3.3.1. Western blot

Western blotting analysis revealed that the expressed IL-4 still retained its antigenicity since it was recognized by the rabbit anti-IL-4 polyclonal antibody. The expressed canine IL-4 was found to be a protein of about 17 kDa occurring in the cell lysate.

Fig. 23: Detection of IL-4 expression by Western blot. Articular chondrocytes were transiently transfected with the pcDNA3.1-IL-4 (lane 1) and non transfected control (lane 2) were lysed with RIPA buffer with protease inhibitors and the extracts probed using IL-4 specific polyclonal antibody. Expression of IL-4 (17 kDa) was detected only in the transfected cells whereas no IL-4 was detectable in the non-transfected cells. As a positive control (3) bacterial expressed his-tag purified recombinant IL-4 was loaded (13 kDa).

It was not detected in mock transfected cells or in cells transfected with empty vector (control pcDNA3.1). Cell lysate from the transfected chondrocytes was harvested 48 h post-transfection, which had been shown to be a suitable time-point for the highest protein production in preliminary studies (Figure 23).

3.3.2. ELISA

Chondrocytes transfected with IL-4 and non-transfected controls were harvested 48 h after transfection and cells were lysed. Samples were subjected to measurement by canine specific IL-4 polyclonal and monoclonal antibodies by using sandwich ELISA. The results from ELISA showed the presence of IL-4 in cell culture lysate with the recombinant IL-4 from *E. coli* as positive control and it was observed that IL-4 antibodies reacted to the IL-4 protein. This protein was detected only in the lysate from chondrocytes transfected with IL-4. No IL-4 levels were detected in normal non-transfected chondrocytes. The measured amount of IL-4 in cell culture supernatant and cell lysate was found to be between 2.6 and 4.3 ng ml^{-1} (Figure 24).

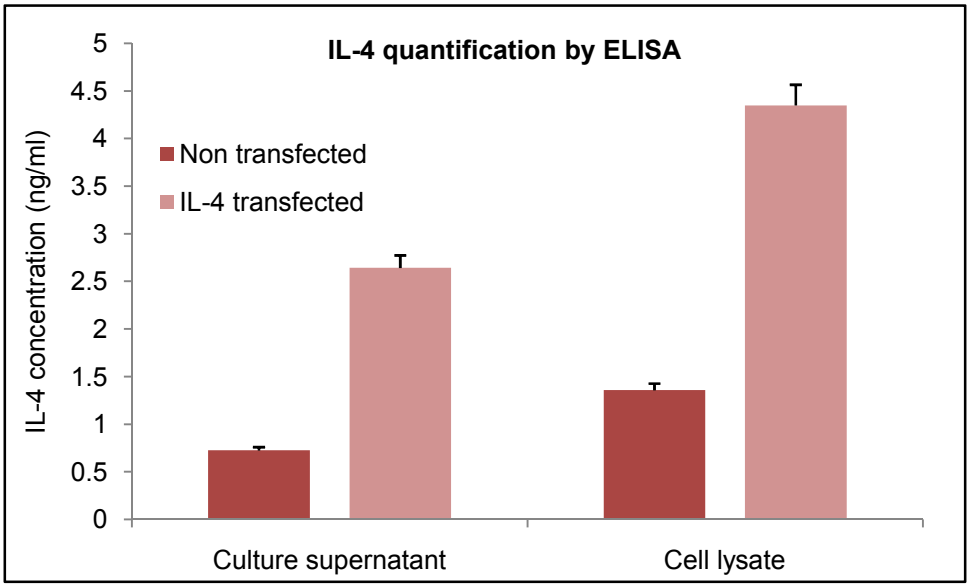

Fig. 24: Detection of IL-4 expression in chondrocytes by ELISA. The plasmid containing IL-4 (pCDNA3.1-IL-4) was transfected into chondrocytes as described in the methods section. After a period of 72 h cells were lysed and the lysates subjected to Western blot using polyclonal antibody raised in rabbits. ELISA yielded a response with cell culture supernatant as well as cell lysate. As shown, the sandwich ELISA using polyclonal antibody as capture antibody and monoclonal mp41as detection antibody. IL-4 protein was detected both in cell culture supernatant as well as lysate from transfected cells.

3.3.3. Effects of IL-4 on inflammatory cytokines

As described above (section 3.2.3) the recombinant canine cytokines IL-1β and TNFα trigger an inflammatory cascade in articular chondrocytes which upon stimulation express more IL-1β and TNFα along with other pro-inflammatory cytokines such as IL-6, 8 as well as enzyme mediators like iNOS. Taking this as a cue, the present study aimed to mimic the inflammatory cascade *in vitro* in articular chondrocytes both in 2D and 3D cultures and to investigate whether the transgene expression of IL-4 from a CMV promoter can down-regulate the inflammatory cytokines, inflammation related enzymes and their products.

To find out whether articular chondrocytes express any cytokines without stimulation, the cDNA from articular chondrocytes with and without stimulation were analyzed by qRT-PCR. The results shown in Figure 25 reveal that cytokine levels in stimulated articular chondrocytes are high when compared to non-stimulated cells. The down-regulation of inflammatory mediators becomes evident when these values are compared with expression levels found in articular chondrocytes that were non-transfected but stimulated with IL-1β and TNFα (Figure 25). The anti-inflammatory effect of IL-4 expressed in chondrocytes is evident for both types of chondrocyte cultures, 2D (Figure 25A) and 3D (alginate) cultures (Figure 25B).

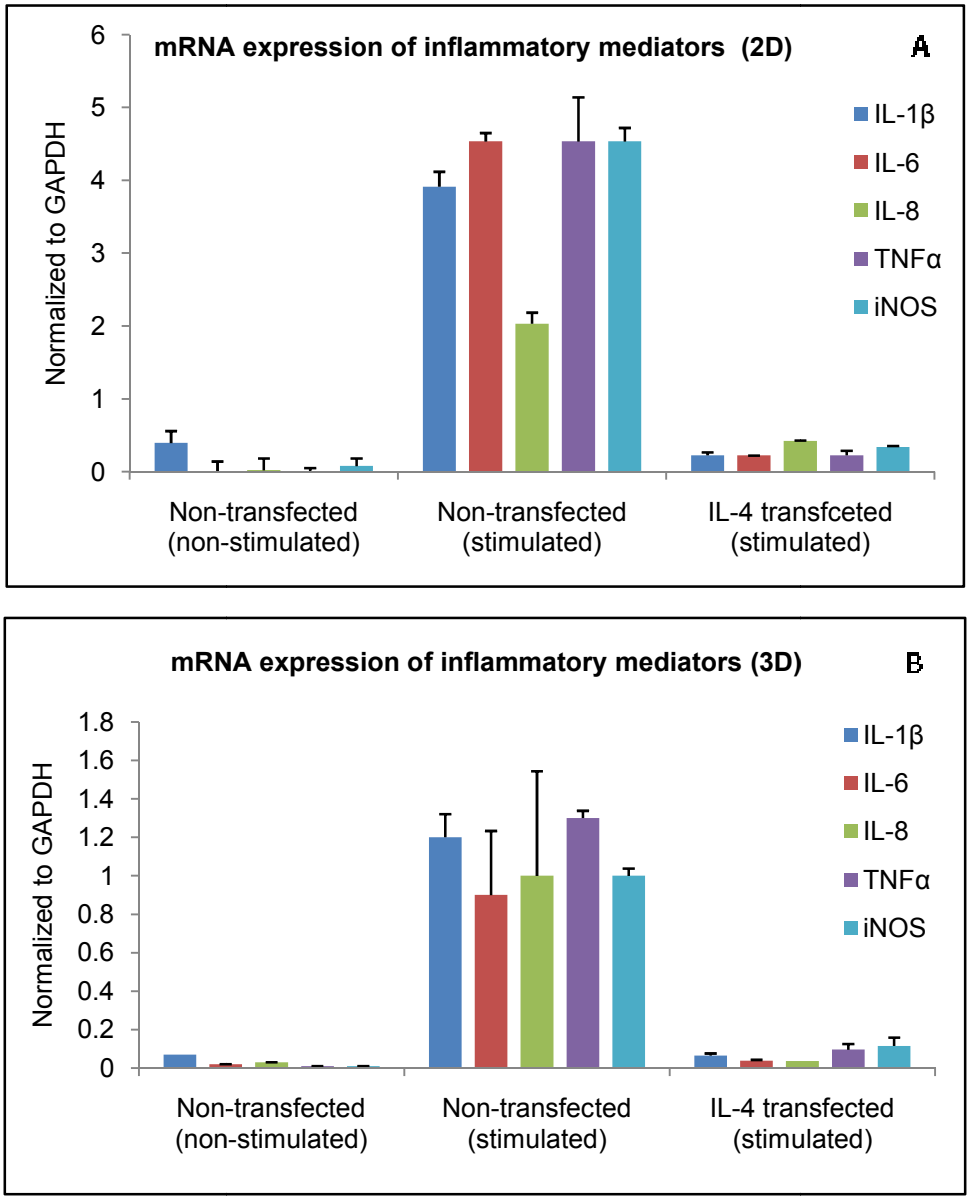

Fig. 25: Effect of IL-4 expression on inflammatory mediators. Articular chondrocytes transfected with pCDNA3.1-IL-4 or non-transfected were stimulated with 100 ng ml^{-1} IL-1β and 50 ng ml^{-1} TNFα in 2D (A) and 3D (B) culture systems. The mRNA expression of IL-1β, IL-6, IL-8, TNFα and iNOS was analyzed using qRT-PCR. Results were normalized using GAPDH as endogenous control and are shown as fold changes relative to untreated controls.

3.3.4. Inhibition of nitrite release by expression of IL-4

As mentioned earlier, NO production via iNOS in articular chondrocytes plays a central role in the pathophysiology of arthritis. In other words, NO production is directly proportional to iNOS production. Nitrite is the one of two stable catabolites of NO. Hence nitrite levels serve as good indicators of inflammation and accordingly,

cell culture supernatants from transfected and non-transfected control cells in 2D and 3D cultures with or without stimulation were subjected to quantification of nitrite levels by the Griess reagent system.

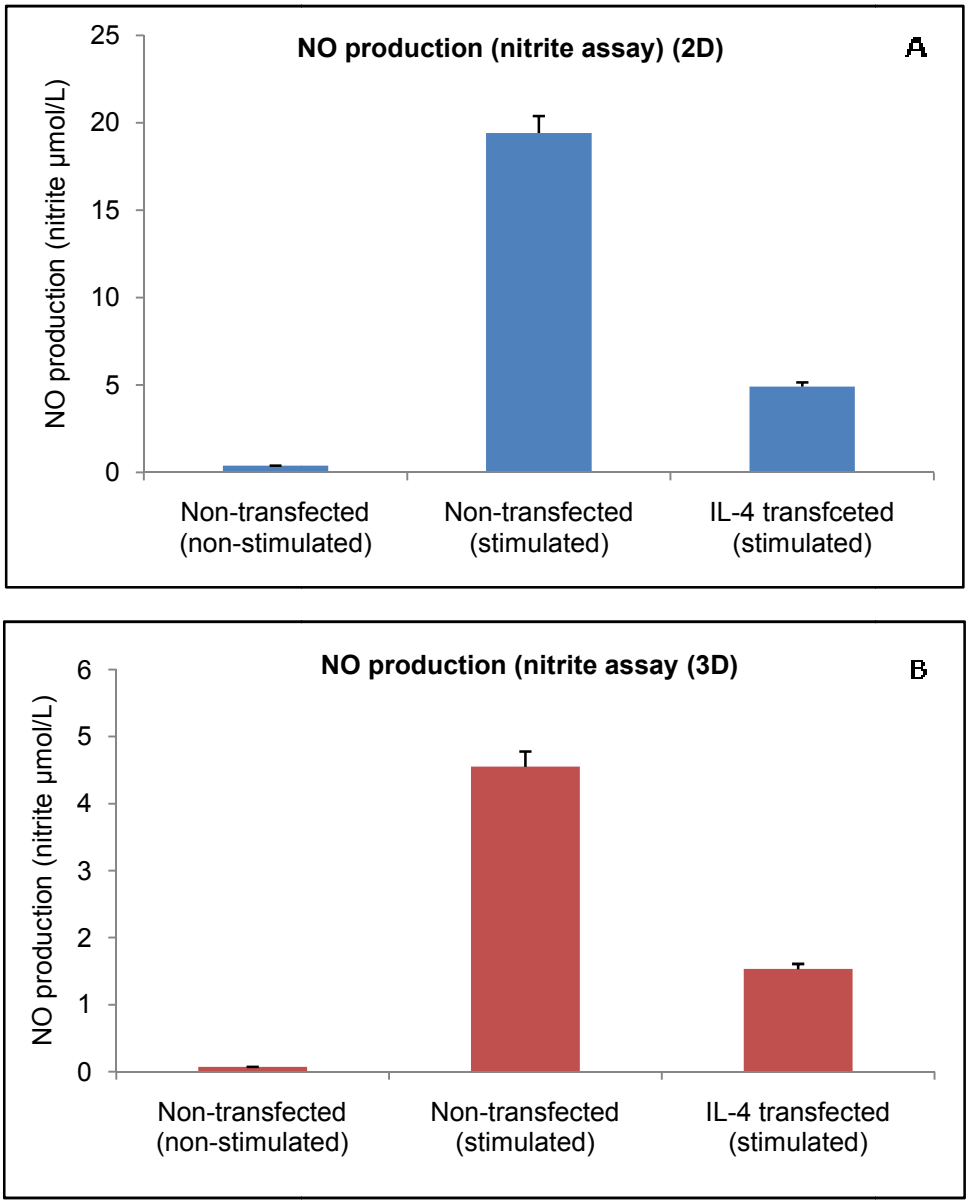

Fig. 26: Effect of IL-4 on nitrite levels. Articular chondrocytes were either transfected with pCDNA3.1-IL-4 or left non-transfected. Cells were either stimulated with 100 ng ml^{-1} IL-1β and 50 ng ml^{-1} TNFα or left without stimulation. As a negative control, non-transfected articular chondrocytes were also cytokine stimulated. Cell culture supernatants from all the samples were assayed for nitrite levels using the Griess reagent system. Inhibition of NO is shown in 2D (A) and 3D (B) culture systems.

As shown in Figure 26 nitrite levels are reduced in the presence of IL-4 in 2D (A) and 3D (B) cultures respectively indicating that iNOS is down-regulated. This is also in accord with the results in Figure 25 which demonstrates that iNOS is down-regulated when IL-4 is being expressed.

3.3.5. Inhibition of PGE_2 production by expression of IL-4

The cell culture supernatant was collected from the transfected and non-transfected control articular chondrocytes with or without stimulation and subjected to PGE_2 quantification by Cayman's EIA kit using PGE_2 monoclonal antibody. Results show that PGE_2 was indeed down-regulated in the samples that were transfected with the constructs containing IL-4. Results shown in Figure 27 were in line with the data obtained from the NO assay and from the quantification of inflammatory cytokines/mediators representing parameters associated with inflammation.

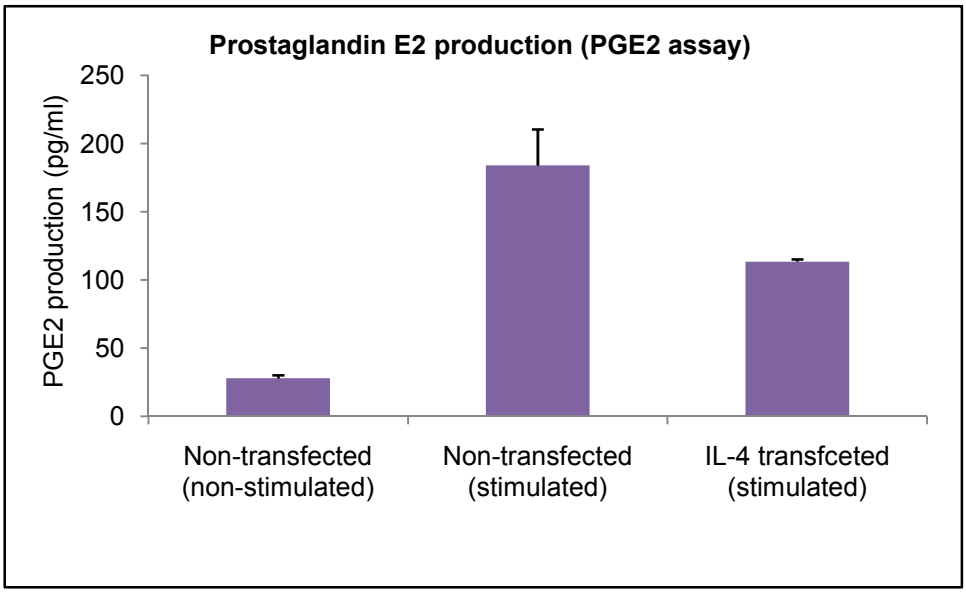

Fig. 27: Effect of IL-4 on PGE_2 levels. Articular chondrocytes were either transfected with pCDNA3.1-IL-4 or left non-transfected. Cells were either stimulated with 100 ng IL-1β and 50 ng TNFα or left without stimulation. Cell culture supernatants from all the samples were assayed for PGE_2 levels using the Cayman EIA kit.

3.3.6. Expression of IL-4 and STAT6

It has been previously reported (Takeda *et al*., 1996; Yu *et al*., 1998) that the effects of IL-4 could be either dependent or independent of STAT6. To answer this question for canine chondrocytes, this work was extended to take a closer look at the signalling cascade utilized by IL-4 in canine chondrocytes. Chondrocytes transfected with pcDNA3.1-cIL-4 or non-transfected (only empty vector) revealed that there was high expressions of IL-4 and STAT6 (Figure 28) only in IL-4 transduced chondrocytes. Non-transfected control showed marginal expression. When the cells were stimulated by exogenous addition of IL-1β and TNFα, the expression of IL-4 as well as STAT6 was upregulated. These finding give support to the idea that signalling from IL-4 in chondrocytes utilizes STAT6 to exert its anti-inflammatory activity.

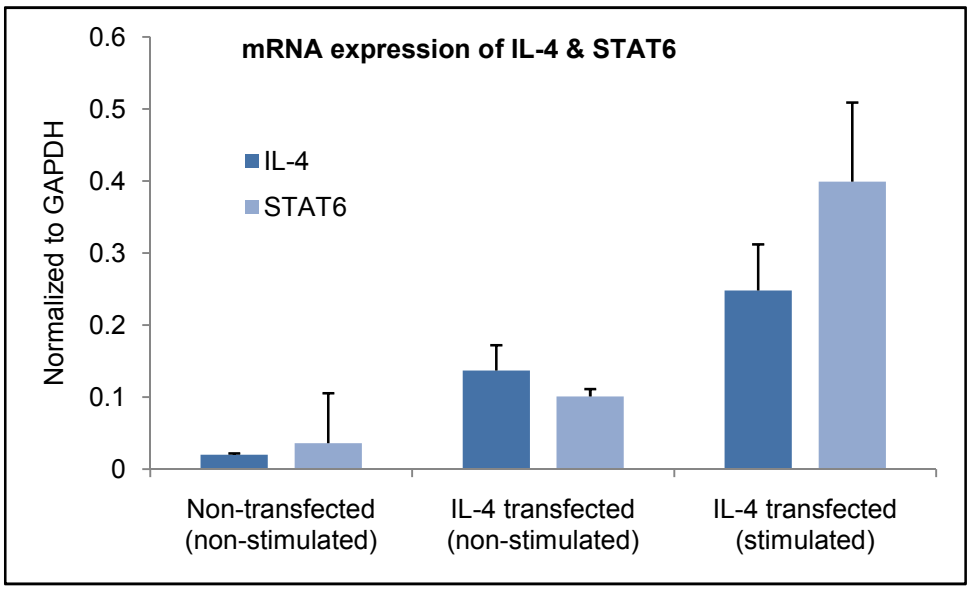

Fig. 28: Expression of IL-4 and STAT6 in chondrocytes. Chondrocytes that were transfected with pcDNA3.1-IL-4 or with empty plasmid vector were lysed for RNA extraction, and samples subjected to qRT-PCR using sequence specific primers. Transfected chondrocytes showed strong signals for IL-4 and STAT6 in transfected cells while samples from non-transfected cells showed only minimal levels of endogenous gene expression. The expression was increased when cell were stimulated with pro-inflammatory cytokines.

3.3.7. Cloning and sequencing of partial STAT6 cDNA

Since canine STAT6 sequences have not yet been published, efforts were made to do gene mining using the human STAT6 gene sequence as a probe. The alignment studies showed that the published STAT6 amino acid sequences from *Homo sapiens* (Accession No. P42226), Mus *musculus* (Accession No. NP_033310), *Bos taurus* (Accession No. BAA96475), and *Rattus norvegicus* (Accession No. NP_001037715) showed more than 85% homology. Hence the choice of human STAT6 as a probe could be justified as STAT6 seems to be conserved among mammalian species.

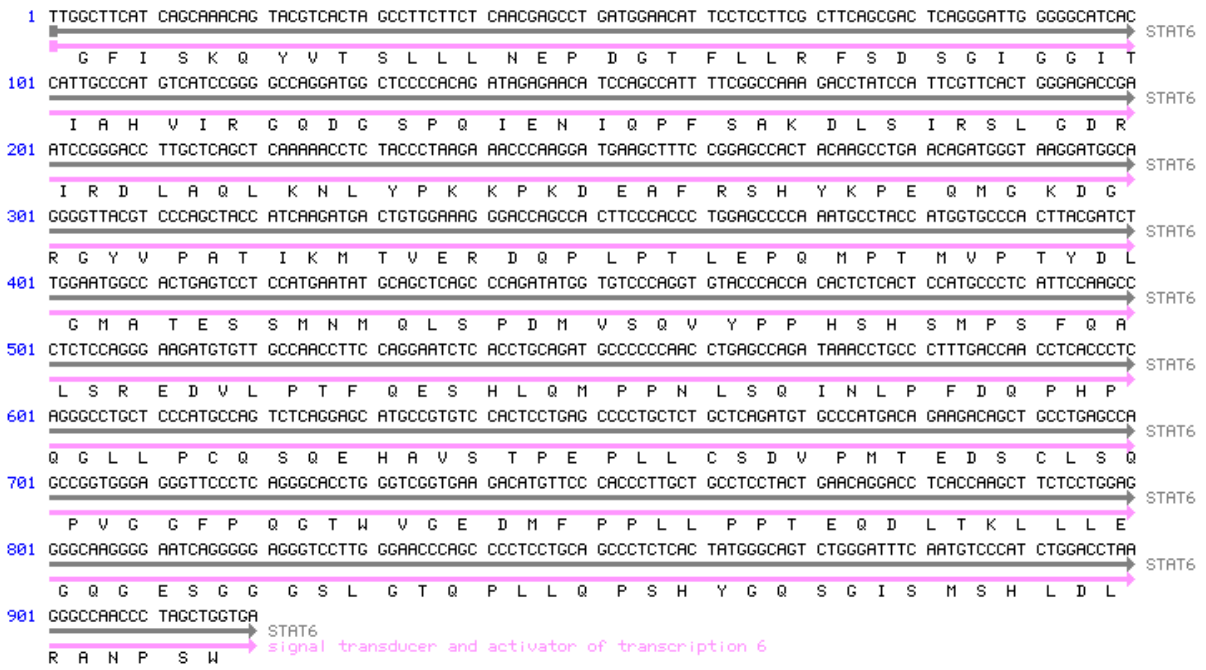

Fig. 29: Partial nucleotide sequence analysis of STAT6. cDNA from chondrocytes transfected with pcDNA3.1-IL-4 was amplified by RT-PCR using sequence specific primers designed from the predicted canine STAT6. The cDNA was cloned in pGEM-T vector and sequenced. Sequence results showed that 920 nucleotides correspond with isoform 2 of the predicted canine STAT6.

In order to obtain a canine specific STAT6 gene sequence, the canine genome database (Accession No. NW_876250; AAEX02029655) using the nucleotide sequence of the human counterpart (Accession No. AF417842) was probed. The resultant gene sequence was intron spliced by use of the web interface software (Augustus gene prediction). Two isoforms were predicted from this method. The difference between these 2 isoforms was that the 2nd isoform spans an additional exon when compared to the 1st isoform. Subsequently, oligonucleotides were designed spanning this region and subjected to PCR on canine cDNA produced from canine chondrocytes. The sequencing results (Figure 29) identified the 2nd isoform as the corresponding gene in canine chondrocytes.

Since no response was obtained from control chondrocytes this STAT6 amplification could only be obtained from canine chondrocytes that were transfected with IL-4. This confirms that the anti-inflammatory properties of IL-4 are mediated through STAT6 expression in canine chondrocytes. In the preliminary experiments, a fragment of approximately 920 bp was cloned and the sequence results showed a 100% homology with a part of predicted STAT6 sequence.

Taking this as a cue, sequencing the full length canine STAT6 by RACE (rapid amplification of cDNA end) and its expression in canine chondrocytes is proposed. The intention is to explore if expression of STAT6 in canine chondrocytes can simulate the effects of IL-4 in down regulating inflammation that has been triggered by IL-1β and TNFα.

4.0. DISCUSSION

This work was conducted firstly to investigate the molecular and phenotypic characteristics and extent of de- and re-differentiation of canine articular chondrocytes in 2D and 3D culture systems. Attempts were made to immortalize chondrocytes by introducing the catalytic component of human telomerase namely hTERT. To determine whether the primary and immortalized cells exhibited the typical articular chondrocyte phenotype, this study examined several morphological, biochemical, and functional features by immunocytochemistry, Western blot and qRT-PCR. Proliferation of cells decreased during cultivation in 2D which was accompanied by their enlargement after P3 and P4. During subsequent cultivation in 2D culture, the expression of collagen type II, aggrecan, CRTAC1, COMP, and Sox9 decreased many fold whereas that of collagen type I increased. Chondrocytes expressing hTERT showed the typical morphology of a cell line. Cells appeared more polygonal and less fibroblastic with decreased doubling time. However, these cells did not express chondrocyte specific genes. Re-differentiation of these immortalized chondrocytes was induced by transferring them from 2D culture to alginate microspheres. As the primary articular chondrocytes, also the telomerase-transduced cells adopted a chondrocyte-specific gene expression pattern within 1-3 weeks in 3D culture. Chondrocytes lose their characteristic phenotype when passaged in 2D culture. Expression of telomerase represents a possibility to expand chondrocytes without the loss of the chondrocyte-specific phenotype in 3D cultures. This may provide a practical tool for testing different tissue engineering applications in the canine model (Rai *et al.,* 2008a).

Second purpose of this work was to develop and characterize 3D model of inflammatory arthritis. Human recombinant IL-1β and TNFα have been previously used to induce a cytokine response in canine chondrocytes. In order to establish this functional relation in a homologous system i.e. canine cytokines in canine chondrocytes *in vitro*, we have developed both 2D and 3D models of inflammatory arthritis using canine recombinant cytokines in canine articular chondrocytes. Canine IL-1β and TNFα were cloned and subsequently expressed in *E. coli*. The purified recombinant canine cytokines were then used to simulate inflammation *in vitro* and the expression of typical inflammation markers such as pro-inflammatory cytokines (IL-1β, IL-6, IL-8, GM-CSF and TNFα), enzyme mediators (MMP-3 MMP-13, iNOS, COX-2) and their catabolites (NO, PGE_2) was measured. High expression of pro-

inflammatory cytokines, enzyme mediators and their catabolites was only observed in IL-1β/TNFα stimulated cells. We conclude that the canine IL-1β and TNFα generated in this study are biologically active and equally effective as human cytokines in the canine cell culture systems. Inducing an inflammatory pathway by canine exogenous cytokines in canine chondrocytes provides a useful tool for the study of canine inflammatory arthritis (Rai *et al.,* 2008b).

Lastly, expression of IL-4 in chondrocytes and its ability to inhibit inflammatory markers in arthritis model were studied. IL-4 is a pleiotropic cytokine with a broad spectrum of biological effects on target cells. This study deals with the mammalian expression of canine IL-4 (IL-4) in canine articular chondrocytes and its ability to down-regulate pro-inflammatory cytokines, enzyme mediators and their catabolites. We transfected IL-4 in chondrocytes which were then stimulated with canine recombinant IL-1β and TNFα or left as untreated control cells. The IL-4 protein was detected by Western blot analysis and quantified by sandwich ELISA utilizing monoclonal and polyclonal antibodies raised against recombinant IL-4. Pro-inflammatory cytokines and enzyme mediators were quantified by quantitative real-time PCR, and nitrite production was measured by a calorimetric assay. These results show that IL-4 is expressed in chondrocytes as a 17 kDa protein, which inhibits various cytokines and inflammatory mediators especially when cells were stimulated by IL-1β and TNFα. Thus, IL-4 expressed in chondrocytes is biologically active and suppresses inflammatory mediators *in vitro*. The expression of STAT6 was similar to that of IL-4. IL-4 transfected chondrocytes showed high expression of both IL-4 and STAT6 and both molecules were upregulated when the transduced chondrocytes were stimulated by exogenous additives (IL-1β and TNFα). It is therefore most likely that IL-4 exerts its anti-inflammatory effects through STAT6 signalling. Following up these findings the canine form of STAT6 was identified which was cloned and partially sequenced (Rachakonda *et al.,* 2008a).

4.1. Molecular and phenotypic characterization of chondrocytes

The advent of regenerative medicine via tissue engineering which, by using allogeneic or autologous cells in combination with a scaffold, has broadened the scope of therapy (Langer and Vacanti, 1993; Endres *et al*., 2007). At the same time, new methods to circumvent the de-differentiation phenomenon and thereby preserving the phenotype of the cells prior to transplantation is of tantamount importance (Minas and Nehrer, 1997; Strehl *et al*., 2002). In this study we have investigated the time course of de-differentiation in 2D culture and the potential for re-differentiation after transfer into an alginate encapsulation system.

The cells after isolation and in 2D culture appeared to change in shape from the initial round/polygonal (early few passages) to elongated fibroblast like cells (Figure 6). Increase in cell size and cellular granularity were found to be passage dependent. The results from Figure 8 indicate that with increasing passage number cell tended to be hypertrophic in the flow cytometry analysis and they shifted towards higher values on the FS channel. A similar trend was observed for cellular granularity. These findings are in agreement with previous report by Fröhlich *et al*. (2007) who found a similar trend in rabbit auricular chondrocytes. Also a significant decrease in growth rate was noted after P3 indicating the initiation of cell senescence. According to the observations made in this study, canine chondrocytes failed to reach confluence subsequent to P7 in 2D culture (Figures 6-7). Simultaneously, we assessed the expression patterns of a series of established chondrocyte specific marker genes in 2D culture in all the passages studied. A comparison was made with those cells that were transferred later than P3 to 3D culture and maintained for a period of one to three weeks. As shown in Figure 11, a typical chondrocyte phenotype is evident in the 3D cultures where the expression of type II collagen, aggrecan, CRTAC1, COMP and Sox9 was re-established and stabilized.

Of all these marker genes, the ratio of type I collagen and type II collagen gives a measure of the extent of the de- and re-differentiation process. While type II collagen is specific for cartilage (Mankin *et al*., 2000), type I collagen is seen in many tissues, including fibrocartilage, bone tendon and skin. Alcian blue staining and immunohistochemistry are established techniques to identify proteoglycan related proteins like aggrecan and type II collagen, respectively. Findings of these experiments indicate a high expression of aggrecan and type II collagen in native cartilage tissue (not shown). As evident from Figures 10-11 type II collagen is

predominant in early passages (detectable only in P1 by Western blot) and its levels dramatically decline below detection by P7. This trend of type II collagen expression correlated with an increase in type I collagen (Salvat *et al.*, 2005). These changes are best documented by calculating the differentiation index of collagen expression which is defined as the CII/CI ratio (Martin *et al.,* 2001; Marlovits *et al*., 2004). In this study, this ratio decreased with passage number in 2D culture and was reversed in 3D culture which showed a steady increase of CII/CI until the third week of encapsulation. The results show (Figure 12) that these changes of expression in 2D culture and vice versa in 3D are gradual and so was the differential index. This observation differs from the findings reported by other authors (Marlovits *et al*., 2004) who reported a constant decrease in the ratio followed by a plateau phase at the end of the culture time. This can be attributed to the fact that these researchers used RNA isolated from pooled cells in culture and therefore the results cannot account for individual differences in the rate of de-differentiation. Furthermore, they explored CII/CI ratio based on numbers of days, the cells remained in culture instead of passage number. The CII/CI ratio can be used to quantify the collagen gene expression in cultured chondrocytes and thus improve the understanding of the process of chondrocyte de- and re-differentiation. Furthermore, this ratio can be used to monitor chondrocytes and their differentiation status in monolayer culture for experimental models of therapeutic use in autologous chondrocyte transplantation (Marlovits *et al*., 2004; Hollander *et al.,* 2006).

The marker genes aggrecan, CRTAC1, COMP, and Sox9 are all inter-related to type II collagen levels and together represent phenotypic conformations of the chondrocytes. Aggrecan is a proteoglycan related protein, while CRTAC1 (previously known as chondrocyte expressed protein or CEP-68) distinguishes chondrocytes from osteoblasts and mesenchymal stem cells in culture (Steck *et al*., 2001; Benz *et al.,* 2002). On the other hand, COMP is a non-collagenous protein and still very important for the assembly, integrity and stability of the ECM synthesized. The interaction of COMP with type II collagen has been previously documented (Rosenberg *et al*., 1998). It is one of the major non-collagenous proteins of the ECM and can be found in articular cartilage (Hedbom *et al.,* 1992). The marker gene Sox9 is a transcriptional factor that has been shown to regulate the expression of type II collagen levels. Sox9 is expressed in all chondroprogenitors and chondrocytes except hypertrophic chondrocytes (Zhao *et al*., 1997). Sox9 is a transcription factor

that is essential for chondrocyte differentiation (de Crombrugghe *et al.,* 2000), and the loss of chondrocyte differentiated characteristics *in vitro* has been linked to the reduced expression of Sox9 (Lefebvre *et al.,* 1998). No data of Sox9 expression in canine chondrocytes is available to date.

The reduction of aggrecan, CRTAC1, COMP and Sox9 expression was manifold during passaging cells from P1-P5. We observed that the expression of the chosen marker genes during re-differentiation was differentially regulated. In the 3D culture system the chondrocytes from normal cartilage tended to maintain their phenotype which was accompanied by the production of primarily type II collagen. Along with this the expression of aggrecan CRTAC1, COMP and Sox9 was re-induced within days after transfer into the three-dimensional matrix. The expression of type I collagen remained undetectable as indicated by qRT-PCR (Grigolo *et al.,* 2002). The results presented here for the canine system emphasize further the plasticity of the chondrocyte-specific phenotype in a 3D *in vitro* culture which has been previously documented and discussed for related systems in other species (Mok *et al*., 1994; Häuselmann *et al*., 1996; Binette *et al*., 1998; Petit *et al*., 1999; Chubinskaya *et al*., 2001). We show that the phenotype of chondrocytes can be modulated by culturing them as 2D on solid surface (cell culture plastic flask) followed by encapsulation in alginate beads. This modification of culture conditions stabilizes the typical synthetic profile of fully differentiated chondrocytes (Bonaventure *et al*., 1994; Liu *et al*., 1998).

While primary canine chondrocytes have a slow growth rate in subsequent passages and have a limited cell passage number, we aimed to generate a chondrocyte cell line by transduction of an hTERT - a human telomerase catalytic subunit. This is based on evidence showing that cell senescence is linked to the length of telomeres (Harley *et al*., 1990), and that incorporation of a transgene expressing telomerase would make the cell immortal (Jiang *et al*., 1999). This concept has been validated in a number of human somatic cell types and chondrocytes, but not in canine chondrocytes. Taking this as a cue, we undertook studies to stably express the human telomerase subunit in chondrocytes by transfection and G418 selection procedures. A comparison was made between the hTERT stabilized cells both in 2D and 3D cultures. Like other immortalized cell line, these cells were also growing indefinitely and attained a complete monolayer (confluence) in 2-3 days. Figure 14 shows the average cell count and growth rate in the newly established GD-hTERT cell line. As shown in the Figure 15 the various chondrocyte marker genes were

better expressed in 3D culture indicating the likelihood of the chondrocyte typical phenotype. The decrease in type I collagen levels in 3D culture was striking compared to 2D. Type II collagen levels were also seen expressed more in 3D, but not on comparable levels as those of other marker genes. However, expression of Sox9 which by far has been recognized as a potent activator of type II collagen (Bell *et al*., 1997; Kypriotou *et al*., 2003) was clearly upregulated in 3D. It was assumed that longer incubation may be needed to achieve even higher type II collagen levels. Similarly, aggrecan levels were seen higher in 3D as were other marker genes. These data show that that hTERT stabilized cell line could substitute normal chondrocytes and moreover provides a reproducible model that mimics the adult chondrocyte phenotype, particularly in 3D culture. Unfortunately, in the preliminary experiments, these cells demonstrated no response to the inflammatory cytokines IL-1β and TNFα unlike primary chondrocytes. Therefore, they could not be used as an inflammation model for cytokine studies. More extensive work is required to characterize these cells and to reveal why they have lost responsiveness to inflammatory conditions during the process of selection. Perhaps the loss of particular surface receptors during the selection procedure could explain this phenotype.

In summary, these results demonstrate that the potential for re-differentiation decreases with increasing passage age of the 2D culture and show that the alginate bead system represents an attractive *in vitro* model to study the chondrocyte de- and re-differentiation processes, as well as ECM assembly. The establishment of an immortalized line of chondrocytes is regarded as a promising start. However, much more detailed characterization of these cells is necessary before they may qualify for use as a tool for the study of inflammation and chondrogenesis *in vitro*.

4.2. Characterization of *in vitro* 3D model of inflammatory arthritis

An *in vitro* cell culture based inflammation model allows studying the effects of anti-inflammatory secretions on pro-inflammatory cytokines. The role of IL-1β and TNFα as the propagators of inflammation leading to cartilage degradation is well documented (Oppenheim *et al*., 1989; Pelletier *et al*., 1991; Westacott and Sharif, 1996; Martel-Pelletier *et al*., 1999). The model presented here, features a homologous system in which canine IL-1β and TNFα are used as tools in canine articular chondrocytes culture. To my knowledge this is the first report attempting to compare this inflammation model in both 2D against 3D cell culture systems. Since 3D culture has been known to revive the type II collagen and aggrecan levels, the present work compared the effects of IL-1β and TNFα on these structural proteins in 2D cultures. At the same time, this study negates the use of bacterial endotoxin lipopolysaccharide (LPS), as they have no direct role in OA pathophysiology. In the absence of commercial canine IL-1β and TNFα (at the time of start of this study), we synthesized these cytokines from canine PBMC after stimulation with the respective human homologue. The respective canine genes without their signal sequences were expressed and purified from an *E. coli* system. Stringent procedures were applied to purify the respective proteins from bacterial cytosolic fractions to rule out contaminants like LPS and other *E. coli* proteins. Additionally, wash buffers included both Triton X-114 and NaCl in order to reduce endotoxin levels to less than 10 $EUmg^{-1}$ (Zimmerman *et al*., 2006). An LAL assay was performed and values of less than 3 $EUmg^{-1}$ indicated the absence of contaminating endotoxin. The absence of contaminating bacterial components was further substantiated by silver staining of purified IL-1β and TNFα proteins after SDS-PAGE. The detection of these proteins using anti-His antibody during Western blot analysis confirmed that the purified proteins were indeed the His-tagged fusion proteins from the expression vector.

To test the biological activity of the purified canine cytokines, the concept from the literature was principally followed that these cytokines should be able to stimulate other inflammatory cytokines, enzyme mediators and their catabolites in cell culture system. The evidence of their functional activity was indicated by the ability of these recombinant proteins to trigger a characteristic inflammation cascade in both 2D and 3D cell cultures. Inflammation was measured by up-regulation of various inflammatory mediators and their catabolites. In showing the production of all major pro-inflammatory cytokines *viz.,* IL-1β, IL-6, IL-8, GM-CSF, TNFα, along with COX-2,

iNOS, MMP-3 and MMP-13 it could be proved that chondrocytes form indeed an ideal choice for *in vitro* studies. In line with published evidence (Kuroki *et al*., 2005), none of the above cytokines and enzyme mediators were seen in the control and non-stimulated cells. We observed that either IL-1β or TNFα alone could trigger a cascade featuring all the above cytokines (not shown). However, their combined use produced higher levels of response indicating the synergistic action of both cytokines in triggering the inflammation cascade (Buchan *et al*., 1988; Shi *et al*., 2004; Zwerina *et al*., 2007). In dose response experiments, we had previously observed that 100 ng ml^{-1} of IL-1β and 50 $ngml^{-1}$ of TNFα represent a good choice of concentrations for the chondrocyte culture system. Time course experiments confirmed that an inflammatory response could be triggered in these cultured chondrocytes from 24 h until 72 h without much variation. While the results obtained from qRT-PCR revealed a minimum two-fold increase in inflammatory marker genes in the stimulated chondrocytes, the calorimetric estimation of inflammation end products such as nitrites (NO metabolism) and PGE_2 (through COX-2 activity) confirmed that the chondrocytes used as the *in vitro* model have all the ingredients for simulation of a complete inflammation cascade.

Another aspect of the present study focussed on the impact of IL-1β and TNFα on cartilage structural proteins i.e., collagen and aggrecan. Results from qRT-PCR show that the levels of type II collagen and aggrecan declined with extended stimulation periods while the levels of MMP-3 and MMP-13 and iNOS steadily increased. This is in agreement with reports which point out that NO along with other mediators such as reactive oxygen species (ROS) leads to cartilage damage (Afonso *et al*., 2007). There are other reports which demonstrate how NO modulates MMPs and COX-2 regulated prostaglandins (Jang and Murrell, 1998). High expression of iNOS and COX-2 correlated with the growing accumulation of both NO and PGE_2 during extended exposure of cells to exogenous IL-1β and TNFα which are indicative for the rising inflammation(Stadler *et al*., 1991; Manfield *et al*., 1996).

However, comparison of the *in vitro* inflammation models in 2D and 3D culture systems revealed that the 2D system is better suited for inflammation studies in which the effect on structural proteins like aggrecan and type II collagen can be neglected. We assume that the bead structure in 3D culture (16000 cells per bead if one million cells are suspended in one ml of alginate solution) could lead to differences in exposure of chondrocytes to the exogenous cytokines (Lemare *et al*.,

1998). It is quite possible that the outer cell layer is more exposed than the inner most cells and therefore better accessible for the inflammatory cytokines added to the culture medium to induce inflammation. Additionally, the need to maintain 3D cell cultures with 10% FCS as compared to 1% FCS in 2D cultures possibly keep the cells more stable and less responsive to stress by exogenous cytokines. Kuroki *et al*. (2005) working with 3D agarose cultured chondrocytes recently reported on a weak response of inflammatory marker genes to exogenous stimulation.

The dominant findings of this present study are that after experimentally inducing inflammation the expression of cytokines and MMPs at all time points was higher than in non-stimulated control cells. It is also noteworthy, that in all the independent experiments there was no significant difference in the expression profile of the cytokines selected for analysis. The consistency of results in both culture systems presents evidence for a successfully established model of inflammatory arthritis in chondrocytes using canine cytokines as the stimulant. Moreover, the patterns of expression obtained support the view that the catabolic effects of IL-1β and TNFα on aggrecan and collagen mediated by MMP expression (Mitchell *et al*., 1996; Lark *et al*., 1997) are mediated through diverse and complex c-jun protooncogene/ AP-1 signal transduction pathways (Geng *et al*., 1996; Mengshol *et al*., 2000). Utilizing this canine model system of inflammation will allow the study of new concepts for the treatment of OA or other diseases with inflammatory complications. Since there is no difference in the principle features of canine and human OA, the model introduced here has high potential for use in veterinary as well as human medical research.

4.3. IL-4 transgene expression in chondrocytes

OA and RA both feature similar inflammatory cascades which culminate in cartilage degradation in chronic conditions. While there is presently no complete cure for these joint diseases, gene therapy with *ex vivo* and non-viral gene transfer especially suits OA conditions. IL-4 is a potent anti-inflammatory cytokine and moreover differentiates naive T helper (Th_0) cells to Th_2 cells. Also it is known to modulate macrophage activity through suppression of inflammatory cytokines (Gautam *et al*. 1992) and is critical for initiating type II immune responses (O'Garra, 1998). The previous clinical studies surmised that the absence of IL-4 in the inflamed synovial fluid of arthritic dogs could have lead to an imbalance of Th_1 /Th_2 cell populations (Wondimu *et al*., 2001; Hegemann *et al*., 2002; Hegemann *et al*., 2005). The role of IL-4 as an anti-inflammatory cytokine in arthritis has been under study for a few species other than canine (van Roon *et al*., 2001; van Lent *et al*., 2002; Leader *et al*., 2006). The interest in novel approaches for the treatment of OA using IL-4 was aimed at the extension of previous studies on IL-4 expression in *E. coli* (Wondimu *et al*., 2001) in this laboratory. The choice of dogs all the same is more befitting, as these animals are naturally susceptible for OA. It has been reported that joint trauma can lead to accelerated or secondary OA (Liossis and Tsokos, 1998) and as such dogs are more prone to joint injuries leading to OA. In addition, OA in dogs can be seen as mimicking the OA of humans and thus studies in dog patients with OA could lead to a better understanding of human OA and facilitate the testing of new strategies for the therapy of OA (and RA). However, *in vitro* simulation of either OA or RA is difficult as they are driven by multiple factors with an involvement of the immune system. Still the inflammatory cascade could be simulated in chondrocyte cultures, and down-regulation of the *in vitro* inflammatory cytokines should serve as a good measure for the efficacy of a therapeutic transgene before proceeding to clinical trials. The results from Western blot analysis confirm that IL-4 expressed in chondrocytes has a similar molecular weight as reported for other species (Chaplin *et al*., 2000; Nuntaprasert *et al*., 2005), which is clearly higher than that of IL-4 expressed in *E. coli*. This expected molecular weight difference in all probability reflects the oligosaccharides due to glycosylation of IL-4 in chondrocytes. Natural glycosylated IL-4 has been reported at a molecular weight of 15-19 kDa (Yokota *et al*., 1986) or 20 kDa (Hamblin, 1989) in human and 19 kDa in mouse (Ramanathan *et al*., 1989). These apparent heterogeneities in size are probably caused by the biochemical modification or

differences in glycosylation of the IL-4 protein after being processed in chondrocytes. The reason for the 20 kDa band consistently occurring with the *E. coli* expressed IL-4 cannot be explained with certainty, although oligomerisation of this expression product to yield higher molecular weight derivatives is a possibility. Since experiments using anti-His tag antibodies (Wondimu *et al*., 2001) identified the lower band as the IL-4 recombinant protein (not shown here), the higher molecular weight band may represent an unrelated *E. coli* protein that could have been co-purified. Expression of IL-4 in chondrocytes occurs between 3-7 days under standard cell culture conditions. While it is quite difficult to quantify the expression of proteins through Western blot analysis, the results from ELISA (Figure 24) reveal that IL-4 is expressed at a minimum concentration of 0.15 ng ml^{-1} culture medium. Thus, approximately $1x10^6$ chondrocytes would secrete more than 2-5 ng total IL-4 protein to the culture supernatants. Previous reports on similar difficulties with IL-4 detection in cell culture supernatant lead to a theory which states that the majority of IL-4 secreted into the supernatant is immediately taken up by the cell through receptor mediated endocytosis and subsequently degraded (Galizzi *et al*., 1989; Friedrich *et al*., 1999; Ewen and Baca-Estrada, 2001). However, the proof of IL-4 expression came from Western blot analysis (Figure 23) of cell lysates from IL-1β /TNFα stimulated articular chondrocytes that were transfected with the respective IL-4 constructs. To my knowledge, this is the first report showing IL-4 detection by Western blot analysis in primary canine cells.

One objective of this study was to assess the anti-inflammatory potential of IL-4 in canine chondrocytes. These results demonstrate that IL-4 in these cells leads indeed to the down-regulation of inflammation. Thus the canine system yields similar results as were obtained with chondrocytes from other species (Roach *et al*., 1989; Olivry *et al*., 1999; van Roon *et al*., 2001; van Lent *et al*., 2002). It was also observed (results not shown) that IL-4 expressed in eukaryotic cells, is functionally more active than the bacterial recombinant IL-4 added to the culture medium of chondrocytes which had been previously induced by IL-1β and TNFα to mimic inflammation. This difference could be attributed to the lack of glycosylation in bacterially expressed IL-4. As in similar systems from other species, IL-1β and TNFα trigger the production of inflammation markers also in chondrocytes. Therefore, it is to be expected that under such conditions one of the end catabolites of the inflammation cascade in OA/RA, NO, should be increased along with super oxide anions and MMP triggered cartilage

degradation. Assessing this end product in cell culture it is demonstrated that the expression of IL-4 indeed inhibits the production of NO (Figure 26) in both culture systems. This finding correlates well with the down-regulation of inducible iNOS which has been shown by qRT-PCR and which is consistent with the latest results from this laboratory where the regulated expression of IL-4 by use of the canine COX-2-promoter is reported (Schmidt and Rachakonda, 2007; Rachakonda *et al*., 2008b). Lastly, efforts were made to trace if IL-4 production triggers STAT6 expression since it has been previously reported that the anti-inflammatory properties of IL-4 are mediated through STAT6 (Ohmori and Hamilton, 1998). The qRT-PCR results show expression of STAT6 in the samples that express the IL-4 gene i.e., articular chondrocytes transfected with IL-4 containing constructs. It was observed that expression of both IL-4 and STAT6 was upregulated in when IL-4 transduced cells stimulated by exogenous additives (IL-1β and TNFα in this case) (Figure 28). A closer look at the signal cascade revealed that IL-4 utilizes STAT-6 which is apparently triggered only after IL-4 expression. This fact indicates that the anti-inflammatory activity of canine IL-4 in chondrocytes is mediated through repression NF-κB (Ohmori and Hamilton, 2000) which is central to the pathogenesis of OA/RA and the associated inflammation. From this study it is evident that IL-4 activates STAT6 and the novel mechanism for IL-4 mediated inhibition of inflammatory gene expression provides an example of a STAT6 involvement (Bennett *et al.,* 1997). Taken together, these data encourage us in suggesting canine IL-4 as a gene therapy candidate which has high potential for the treatment of inflammatory joint diseases in canine patients.

4.4. Conclusion and future prospective

One of the biggest challenges facing gene therapy today is the improvement of current vectors and this area continues to be the major limiting factor in gene therapy applications. Although the current adenoviral vectors are suitable for long-term transgene expression when administered in conjunction with immunomodulating agents, obvious problems exist with this approach. Researchers in the field of vector engineering have realized these problems and have focussed significant effort into a solution.

A large amount of new information has been generated in the past few decades that provides guidance in the development of new and novel therapeutic strategies to delay the progression of the structural changes associated with OA. A comprehensive therapeutic intervention in OA should integrate a clear understanding of the major pathophysiological factors that contribute to the progression of the disease at both the clinical and molecular level. The current use of gene therapy in the treatment of OA is a result of success in identifying the major pathophysiological pathways of the disease process. The principle, which underlies gene therapy, is that a disease can be treated by controlling the expression of a number of genes that are responsible for the synthesis of factors involved in cartilage degradation (catabolic) and/or those that promote cartilage repair (anabolic) (Gelse *et al*., 2005). At this point inflammation can be reduced which could lead to the moderation of pain. The rationale for the use of gene therapy strategies in OA is that, in addition to providing a more effective and sustained delivery of therapeutic agents. These molecules (proteins) are delivered to a precise location. For joint tissues, it has been possible to transfer genes by indirect methods using host cells or by direct transfer using plasmid DNA constructs with different types of carriers to improve efficiency. Until now, gene transfer to synovium has been more successful than gene transfer to cartilage.

The results presented in this dissertation provide a practical framework to test a novel cytokines-responsive promoter construct in cultured chondrocytes and in an essential 3D model for *in vitro* studies of inflammatory arthritis where delivery of anti-inflammatory cytokines or of biological can be guaranteed.

Further elucidation of the effects of these pro-inflammatory cytokines on chondrocytes is crucial not only for the further understandings of the disease mechanisms of OA but also for establishing effective and safe treatment modalities. Although the identification of a sole etiological factor for the development of OA in

humans and in companion animals is unlikely, studies that focus on the investigation of cellular characteristics of articular chondrocytes may deepen the understanding of the disease and provide vital information and efficacious treatment of OA.

4.0. REFERENCES

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PUBLICATIONS

A. From the present work

I. Peer reviewed Publications

1. Rachakonda PS, **Rai MF,** Schmidt MFG. 2008. Application of inflammation-responsive promoter construct to reduce inflammation markers in an *in vitro* model. *Arthritis Rheumatism* 58(7): 2088-97.

2. **Rai MF,** Rachakonda PS, Manning K, Vorwerk B, Brunnberg L, Kohn B, Schmidt MFG. 2008. Quantification of cytokines and inflammatory mediators in a three-dimensional model of inflammatory arthritis, *Cytokine* 42(1):8-17.

3. Rachakonda PS[*], **Rai MF[*],** Manning K, Schmidt MFG. 2008. Expression of interleukin-4 (IL-4) in canine articular chondrocytes (CAC) inhibits inflammatory cascade through STAT6. *Cytokine* (in press). [*]*Both authors contributed equally to this work.*

4. **Rai MF,** Rachakonda PS, Manning K, Palissa C, Sittinger M, Ringe J, Schmidt MFG. 2008. Molecular and phenotypic modulation of primary and immortalized canine chondrocytes in different culture systems. *Cell & Tissue Research* (submitted).

II. Sequences submitted to EMBL/GenBank/DDBJ databases

1. Rachakonda PS, **Rai MF,** Manning K, Schmidt MFG. **2008.** *Canis familiaris* signal transducer and activator of transcription 6, interleukin-4 induced (STAT6) - mRNA partial-cds. GenBank Accession No. EU439612.

2. Vorwerk B, Rachakonda PS, Manning K, **Rai MF,** Schmidt MFG. 2008. *Canis familiaris* interleukin 10 - mRNA complete-cds. GenBank Accession No. EU426968.

3. Rachakonda PS, **Rai MF,** Schmidt MFG. 2007. *Canis familiaris* Cyclooxygenase -2 (COX-2) truncated promoter and part of the 5' UTR – GenBank Accession No. EU249362**.**

4. **Rai MF,** Rachakonda PS, Vorwerk B, Schmidt MFG. 2007. *Canis familiaris* interleukin-1β - mRNA complete-cds. GenBank Accession No. EU249360 (NM_001037971)**.**

5. **Rai MF,** Rachakonda PS, Vorwerk B, Schmidt MFG. 2007. *Canis familiaris* tumor necrosis factor α - mRNA complete-cds. GenBank Accession No. EU249361 **(**NM_001003244**).**

III. Posters/abstracts

1. **Rai MF,** Rachakonda PS, Schmidt MFG. 2008. IL-4 expression under the control of the COX-2 promoter suppresses inflammatory mediators in response to disease conditions. *The Annual European Congress of Rheumatology,* Paris-France. Annals of the Rheumatic Diseases 67: 456.
2. Manning K, **Rai MF,** Schmidt MFG. 2008. Enhanced regenerative effects in canine chondrocytes *in vitro* upon dual expression of IGF-1 and IL-4. *The Annual European Congress of Rheumatology,* Paris-France. Annals of the Rheumatic Diseases 67: 465.
3. Rachakonda PS, **Rai MF,** Manning K, Schmidt MFG. 2006. Promoter squelching as a tool to downregulate the inflammatory cascade in canine Osteoarthritis. 2006 World Congress on Osteoarthritis, Prague, Czech Republic. *Osteoarthritis & Cartilage,* 14: S135.

B. Previous peer reviewed articles

1. Ahmad MD, Chaudhry M, **Rai MF,** Rashid HB. 2007. Evaluation of Two Vaccination Schemes Using Live Vaccines against Newcastle Disease in Chickens. *Turkish Journal of Veterinary & Animal Sciences*. 31:165-9.
2. **Rai MF,** Maqbool A, Tanveer A, Tipu MY. 2005. Effect of inoculating *Salmonella gallinarum* into yolk sac on health status of broiler chicks. *Punjab University Journal of Zoology*, 20:61-6.
3. **Rai MF,** Khan SA, Aslam A, Saeed K. 2005. Effects of yolk sac infection in chicken. *Avian & Poultry Biology Reviews* 16:87-93.
4. Shah MS, Khan SA, Aslam A, Rabbani M, Khan KA, **Rai MF.** 2004. Effect of experimental yolk sac infection with *Escherichia coli* on immune status of broiler chicks. *Pakistan Veterinary Journal*, 24:125-8.
5. Hussain MI, Khan SA, Chaudhary ZI, Aslam A, Ashraf K, **Rai MF.** 2004. Effect of organic and inorganic selenium with and without vitamin E on immune system of broilers. *Pakistan Veterinary Journal*, 24:1-4.

6. Khan MS, Khan SA, Chaudhary ZI, Khan MN, Aslam A, Ashraf K, Ayyub RM, **Rai MF.** 2004. Mercury intoxication in Grass carp *(Ctenopharyngodon idella) Pakistan Veterinary Journal*, 24:33-8.

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8. **Rai MF,** Ahmad MD, Tanveer A, Maqbool A, Ahmad RS. 2004. Studies on the immunopathology and haematology of broilers experimentally infected with *Escherichia coli. Punjab University Journal of Zoology*, 19:51-7.

9. Ahmad MD, Rauf A, **Rai MF.** 2004. *E. coli* infection and antibiotic resistance in diseased and healthy broilers. *Online Journal of Veterinary Research,* 8:79-84.

10. Dar EM, Khan MA, Mehmood AK, Saeed K, **Rai MF.** 2004. Effects of experimentally induced intestinal obstruction on the electrolyte profile in dogs. *Science International* (Lahore), 16:143-6.

11. **Rai MF,** Khan SA, Aslam A, Saeed K, Khan KA, Shah MS. 2003. Effect of experimental yolk sac infection with *Staphylococcus aureus* on immune status of broilers. *Pakistan Veterinary Journal*, 23:84-90.