2014-04-04

The Role Of Inflammation In A New Rabbit Model Of Post-Traumatic Osteoarthritis

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The Role Of Inflammation In A New Rabbit Model Of Post-Traumatic Osteoarthritis

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF MEDICAL SCIENCE

CALGARY, ALBERTA

MARCH, 2014

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Cy Frank for all his help, guidance and patience throughout my PhD. I would also like to thank my committee: Dr. Shrive, Dr. Hart and Dr. Kubes for all their assistance, my external examiners: Dr. Christopher Evans and Dr. Andrea Clark and my candidacy committee: Dr. Fritzler and Dr. Buret for their wise questions and helping me understand the pathophysiology and larger context of OA and inflammation.

I would also like to thank my friends and family for all their love and understanding. And to my fellow Medical School classmates (you know who you are) that have been so supportive and helped me through the craziness of completing this joint program.

Lastly, the lab and department – May Chung, Bryan Heard, Etienne O'Brien, Nathan Solbak, and Jill Beveridge. Your friendship and support were there on a daily basis. Thank you for not only your collegiality but your friendship.

This work was supported through funding provided by the Canadian Arthritis Network, Canadian Institutes of Health Research, Alberta Innovates Health Solutions, and the Leaders In Medicine program (Studentships).

PUBLISHED WORKS NOT PRESENTED IN THIS THESIS

O'Brien EJ, Beveridge JE, **Huebner KD**, Heard BJ, Tapper JE, Shrive NG, Frank CB. Osteoarthritis developed in the operated joint of an ovine model following ACL reconstruction with immediate anatomic reattachment of the native ACL. *J Orthop Res. 2013 Jan; 31(1): 35-43.*

Frank CB, Beveridge JE, **Huebner KD**, Heard BJ, Tapper JE, O'Brien EJ, Shrive NG. Complete ACL/MCL deficiency induces variable degrees of instability in sheep with specific kinematic abnormalities correlating with degrees of early osteoarthritis. *J Orthop Res. 2012 Mar; 30(3): 384-92.*

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PREFACE

Based on the fact that some of my work has already been published and some is in preparation for publication, this thesis is structured as a series of manuscripts but with a more typical monograph-type abstract, introduction and conclusion. The abstract is followed by an introduction in chapter 1, which summarizes the current literature on topics that are relevant to my work. Chapters 2 through 5 each contain an abstract, introduction, methods, results, figures and tables and discussion for a specific set of experiments. The development of a novel surgical model of post-traumatic osteoarthritis (PTOA) in which joint mechanics were not altered and only the biological joint environment is disrupted is presented in Chapter 2; this chapter has been published - Huebner KD, Shrive NG, Frank CB. New surgical model of post-traumatic osteoarthritis: Isolated intra-articular bone injury in the rabbit. *J Orthop Res*. 2013 Feb 19. [Epub ahead of print]. Chapter 3 shows through the effectiveness of dexamethasone in inhibiting PTOA that inflammation is a key component of the pathogenesis of osteoarthritis in my model (Accepted to *J Orthop Res)*. After demonstrating that inhibiting inflammation prevented cartilage damage, IL-1Ra (Chapter 4) and tripeptide feG (Chapter 5) were tested as targeted anti-inflammatories to prevent post-traumatic osteoarthritis. Chapters 6-9 contains an overall discussion of results, limitations, conclusions and future directions for chapters 2 through 5. A list of referenced literature is followed by appendices including supplementary tables, copyright permissions, and an unrelated study of anterior cruciate ligament ultrastructure during ovine maturation - Huebner KD, O'Brien EJ, Heard BJ, Chung M, Achari Y, Shrive NG, Frank CB. Post-natal molecular adaptations in anteromedial and posterolateral bundles of the ovine anterior cruciate ligament: one structure with two parts or two distinct ligaments? *Connect Tissue Res.* 2012;53(4):277-84.

ABBREVIATIONS

ACL:	anterior cruciate ligament
ACR:	American college of rheumatology
AGE:	advanced glycation end products
AM:	anteromedial
ANOVA:	analysis of variance
BPI:	bactericidal permeability increasing protein
bFGF:	basic fibroblast growth factor
BI/ Drill/Drill Sx:	bone injury/intercondylar notch drill surgery
BMP:	bone morphogenic protein
Ca^{++}:	calcium
CI:	confidence intervals
Ctl:	controls
COX:	cyclooxygenase
DEX:	dexamethasone treated animals
ECM:	extracellular matrix
EDTA:	ethylenediaminetetraacetic acid
Eo:	eosinophils
ERK:	extracellular signal-regulated kinases
FG:	femoral groove
FGF:	fibroblast growth factor
GAG:	glycosaminoglycans
GAPDH:	glyceraldehyde 3-phosphate dehydrogenase
GDF5:	growth differentiating factor 5
HMGB:	high mobility group box domain
HRP:	horse radish peroxidase
HSD:	honestly significant difference
ICAM:	intercellular adhesion molecule
IFN:	interferon
IGF:	insulin-like growth factor
IgG:	immunoglobulin G
IL:	interleukin
IL-1β:	interleukin 1 beta
IL-1Ra:	interleukin 1 receptor antagonist
IRF:	interferon regulatory factors
IV:	intravenous
K^{+}:	potassium
KOOS:	knee OA outcome score
LFA:	leukocyte function associated molecule
LFC:	lateral femoral condyle
LIF:	leukemia inhibitory factor
LPS:	lipopolysaccharide
LSD:	least significant difference
LTP:	lateral tibial plateau
Mϕ:	macrophages
MANOVA:	multivariate analysis of variance

MC:	mast cells
MCL:	medial collateral ligament
MCP1:	monocyte chemoattractant protein 1
μCT:	micro-computer tomography
MD:	mean difference
MFC:	medial femoral condyle
MFD:	mean fibril diameter
MIA:	monosodium iodoacetate
MIF:	macrophage inhibitory factor
MMP3:	matrix metalloproteinase 3
MMP13:	matrix metalloproteinase 13
mRNA:	messenger ribonucleic acid
MSCs:	mesenchymal stem cells
MTP:	medial tibial plateau
NALP:	nucleotide-binding oligomerization domain containing protein-like receptors
NETs:	neutrophil extracellular traps
NFκB:	nuclear factor kappa-light-chain-enhancer of activated B
$NO^{\cdot}$:	nitric oxide
NOD:	nucleotide-binding oligomerization domain containing protein
NSAIDs:	non-steroidal anti-inflammatories
NZW:	new Zealand white
OA:	osteoarthritis
OCT:	optimal cutting temperature
PAF:	platelet activating factors
PDGF:	platelet derived growth factor
PGE/PGI:	prostaglandin E/I
PKC:	protein kinase C
PL:	posterolateral
PMN:	polymorphonuclear cells/neutrophils
PRP:	platelet rich plasma
PT:	patellar tendon
PTOA:	post-traumatic osteoarthritis
qPCR:	real time reverse transcriptase polymerase chain reaction
RA:	rheumatoid arthritis
RAGE:	receptor for advanced glycation end products
ROS:	reactive oxygen species
SDS-PAGE:	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sham:	sham surgical animals
SMR:	submandibular gland rat
SC:	subcutaneous
TEM:	transmission electron microscopy
TGFβ:	transcription growth factor beta
TIMP:	tissue inhibitory metalloproteinase
TLR:	toll-like receptors
TNF:	tumor necrosis factor

T-PER:	tissue protein extraction protein
WOMAC:	Western Ontario and McMaster universities Arthritis index

ABSTRACT

Osteoarthritis (OA) is a debilitating chronic disease. A significant proportion of the population suffering from OA develop it post-injury. This is particularly worrisome as this population is often young, and current interventions do little to prevent or delay the development of OA. Anterior cruciate ligaments (ACL) are commonly damaged during injuries, and reconstruction fails to prevent subsequent development of OA. Many studies are assessing mechanical changes to the joint after injury as the cause of OA; however, if mechanics are maintained after surgical intervention, biological changes in the joint must be contributing to the development of OA. Inflammation has become an area of interest in the pathogenesis of OA. There is little understanding of the pathophysiology of the development of post-traumatic OA (PTOA) and without this, few interventions will be successful in preventing OA. Using a novel animal model of PTOA developed and validated for this purpose, which produced no gross mechanical instability, I tested the hypothesis that after an isolated intra-articular bone injury, inflammation is critical in the development of PTOA.

The major findings of this dissertation confirm this central hypothesis and are as follows: First, intra-articular drill holes reproducibly result in cartilage damage as seen in OA without gross mechanical instability. Second, the use of intra-articular glucocorticoids inhibits inflammation and prevents cartilage damage in this model, confirming the role of inflammation in causing damage. Third, IL-1Ra partially inhibits synovial and fat pad inflammation and partially protects articular cartilage. Fourth, feG which inhibits leukocyte migration is ineffective in inhibiting joint inflammation and cartilage damage, suggesting leukocyte migration alone is not a major factor in OA.

These studies suggest that PTOA has a significant inflammatory component resulting in cartilage damage. While my work suggests that post-injury use of glucocorticoids may have a

role in inhibiting cartilage damage, further study of the specific inflammatory mechanisms and the use of more targeted anti-inflammatory treatments will elucidate better management of joint injuries and provide a framework for not only better understanding PTOA, but for preventing its development.

Chapter 1. INTRODUCTION AND LITERATURE REVIEW

Osteoarthritis (OA) is the most common chronic disease of joints. It currently affects 1/8 Canadians and it has a significant impact on patients' quality of life, societal disability, and cost. In its 2011 report, the Arthritis Alliance of Canada reported that OA had a negative economic impact of $2.75 billion dollars due to productivity losses in Alberta alone. Joint injuries are a common cause of OA and this type appears to be increasing in society as injuries to the joints that destabilize them, such as injuries to the anterior cruciate ligament (ACL), are apparently now occurring more frequently. In fact, the development of post traumatic OA (PTOA) has long been thought to be caused by mechanical damage after the joint injury, presumably caused by on-going instability and abnormal joint stresses. However, in the last twenty years, inflammation has emerged as another possible contributor to the pathogenesis of OA. Although the body of work in this field is increasing, the precise inflammatory mechanisms in OA are still not well understood. One of the challenges in understanding the pathophysiology of OA is limited by current animal models, which either do not represent human physiology or they destabilize the joint, thus failing to allow the separation of mechanical and biological causes of secondary joint damage. The studies in this dissertation examine the more isolated impact of inflammation on joint cartilage is a new rabbit model of PTOA which mimics some of the physiological conditions of a joint injury without grossly destabilizing the joint.

1. KNEE FUNCTION, ANATOMY AND COMPOSITION

As one of the most important weight bearing joints and a joint used in movement, the knee transfers large forces from the thigh to the lower leg during locomotion. The knee is made up of a complex bone and soft tissue apparatus that provides load transmission to the ground but with mechanical stability to the joint as it moves through a complicated range of three dimensional motions (1). The knee is a self-contained organ system consisting of two major boney articulations (patellofemoral and tibiofemoral) and it contains three interconnected but anatomically distinct functional joint compartments: the patellofemoral joint, the medial compartment, and the lateral compartment; each defined by its three main bones - patella, femur and tibia (2). There are several intra-articular structures that give the knee its unique structure and functions, these include: two ligaments (the anterior cruciate ligament (ACL) and the posterior cruciate ligament (PCL)), two menisci (the medial and the lateral – not discussed in this thesis) and the hyaline articular cartilage. Synovium (a thin layer of tissue that lines the entire joint), synovial fluid (that lubricates the entire joint) and the fat pad (that cushions the front of joint and helps distribute synovial fluid anteriorly) are also key intra-articular components of the joint (3-6). See Chapter 1, Figure 1 for a schematic representation of normal joint anatomy with the joint capsule removed.

From the perspective of OA, an important tissue to understand is the articular cartilage as it is the protective surface layer of the bones that deteriorates during the processes of OA, eventually leaving the highly innervated bones unprotected. Normal articular cartilage that covers all articulating surfaces in the joint is grossly white, thin, firm, and extremely slippery. Interestingly, it is moderately compressible and it does deform under load as it is composed mainly of water with a number of highly specialized tissue components that make up its viscoelastic matrix.

The most common protein in the articular cartilage matrix is collagen type II, which is dispersed through the ECM (extracellular matrix) and is organized histologically in 4 distinct zones. The most superficial and thinnest layer is the tangential zone which contains fibers which lie parallel to the joint surface, providing a tough membrane resistant to tensile stresses (7, 8). The next layer, the thickest layer, is the middle zone. In that zone, collagen fibers are distributed more loosely and parallel to the surface to resist stresses from joint loading (7). The deep zone consists of fibers oriented radially forming bundles that cross the tidemark into the innermost layer, the calcified zone, to anchor the cartilage to the subchondral bone (9). Chondrocytes make up the cell population of the articular cartilage, which are distributed throughout making cartilage essentially hypocellular, avascular and aneural (10).

The second largest component of the articular cartilage is the proteoglycan (PG) known as aggrecan, which binds to glycosaminoglycans (GAGs). Crosslinks between aggrecans and GAGs (mainly hyaluronic acid) impart structural rigidity and strength to the cartilage (11) which in addition to conformational changes and shortening is lost with aging (12). Immobility also decreases the content and alters the conformation of proteoglycans (13). Proteoglycans are also very important for holding the articular cartilage together. Water makes up the majority of wet-weight of cartilage which maintains tissue hydration in loading states, contributes to joint lubrication, and allows the transport of nutrients and waste between the synovial fluid and the cartilage. Due to the structure and functions of these various components that work together in interesting ways within the tissue itself, cartilage can withstand high loads and respond uniquely to various mechanical conditions which will not be discussed in this thesis.

The fat pad and synovium are also critical in the development of OA. The synovium and fat pad unlike the cartilage has significant vasculature, which transfers nutrients to the nearby cartilage and menisci (14). The synovium is composed of collagen type I mainly with a

heterogeneous subset of cells. Mononuclear cells including macrophages are distributed throughout the synovium (15), while fibroblast-like synoviocytes are the most abundant cells in the stroma producing the tissue scaffolding (16). These synoviocytes transition to myofibroblasts in disease expressing various proteases (17). The fat pad is composed of packed adipocytes covered by a layer of synovial cells (18).

The knee is much more than a simple hinge joint, having 6 degrees of freedom: 3 translations (anterior-posterior, lateral-medial, superior-inferior) and 3 rotations (flexion-extension, internal-external rotation, abduction-adduction). The soft tissues, primarily the ligaments, are responsible for the stability of the joint, with secondary stability coming from the menisci (19). The ligaments are composed primarily of collagens (type I, III, IV, V, and VI) produced by fibroblasts. They also contain decorin, chondroitin sulfate, keratin sulfate, elastin, fibrillin, fibronectin, laminin, thrombospondin and tenascin. ACLs have a bimodal distribution of collagen fibrils (40-75nm and 100-150nm) that are cross-linked forming a characteristic crimp pattern (20). The cellular makeup of ligaments includes fibroblasts, mast cells, nerve cells, and endothelial cells (21). The ACL prevents anterior translation of the tibia on the femur and provides rotational stability of the knee. The anterior cruciate ligament (ACL) is commonly injured, and has often leads to PTOA. After an injury, joint mechanics are altered (22), patients are unable to regain pre-injury performance, often unable to return to full activity levels and frequently re-injure their knees (23, 24). Even more worrisome is that reconstruction of ACL does not necessarily prevent this series of events and over 50% of those with ACL reconstructions still appear to be going on to PTOA.

When discussing OA it is also important to consider the vasculature and nerves in the knee joint. As described above the cartilage and meniscus are relatively avascular, therefore relying on nutrients from surrounding tissues like the fat pad, synovium and subchondral bone

(14, 25). The ligaments have a heterogeneous vascularization, receiving their blood supply from braches off the popliteal artery as well as from synovial vessels. However, due to their fibrocartilaginatous attachments the ends of ligaments are relatively avascular (21). Joint innervation parallels it's blood vessels, with avascular structures also being aneural (14). Non-myelinated sensory nerves accompany blood vessels in the joint. The synovium, ligaments and subchondral bone contains substance P and calcitonin gene-related peptide 1 perivascular nerves (26-28). The synovial sympathetic nerves also contain neuropeptide Y similar to other sensory nerves (26).

2. INFLAMMATION

At a tissue level, inflammation is a response of the microcirculation in which fluid and leukocytes migrate from the blood into the extravascular tissue. Considerable advances have been made in understanding the cellular and molecular events in the acute inflammatory response. Much less is known about chronic inflammation that occurs in a variety of diseases, for example osteoarthritis.

Inflammation was first described by Aulus Celsus as redness, heat, swelling, and pain. A fifth clinical sign, a loss of function, was added by Rudolf Virchow, student of Cohnheim. Julius Cohnheim used the microscope to view inflamed blood vessels in the mesentery and tongue of a frog. He noted immediate changes in blood flow, and subsequently vascular permeability causing edema, and leukocyte recruitment (29). Eli Metchnikoff and Paul Ehrlich concluded that the purpose of inflammation was to bring phagocytic leukocytes to injured tissue. The importance of chemical mediators, such as histamine in the inflammatory response to increase vascular permeability and leukocyte migration was described by Sir Thomas Lewis.

2.1. Overview of inflammation

Inflammation is thought to proceed as follows: initiation of recruitment and clearance factors, amplification of the inflammatory response, and termination of the inflammatory response. In some conditions the ability to regulate inflammation is altered, and inflammation is harmful to the patient leading to excessive tissue damage. Initiation of inflammation starts within the microvasculature, specifically the post-capillary venules which contain platelets and leukocytes. After tissue injury there is a loss of endothelial cell integrity, leakage of fluid from intravascular tissue, and emigration of leukocytes. For transducers and mediators of inflammation see Chapter 1, Figure 2.

2.1.1. Inducers

The components of the inflammatory response can be divided into inducers and mediators. Inducers are signals that initiate inflammation; these can be exogenous or endogenous. Exogenous inducers are either microbial via pathogen-associated molecular pathways (PAMPs) or virulent factors or non-microbial via allergens, irritants, foreign bodies and toxins. Endogenous signals are produced by stress, tissue damage, or tissue that is not functioning.

Inflammation resulting from microbial infections and tissue injury are triggered by innate immune receptors, Toll-like receptors (TLRs) and NOD (nucleotide-binding oligomerization domain containing protein)-like receptors (NLPs) which are mediated by trolling mast cells (MCs) and macrophages (Mϕ) (30, 31). These cause the release of chemokines, cytokines, vasoactive amines and eicosanoids which activate the endothelium. The activated endothelium presents selectins and immunoglobulin (Ig) proteins causing extravasation of neutrophils (PMNs) through the presentation of integrins and chemokines (32). Once PMNs reach the target site they become active, killing invading pathogens or damaged tissue by releasing granules, releasing reactive oxidase species (ROS), releasing nitric oxide, producing proteinases, and extruding neutrophil extracellular traps (NETs) (33, 34). Next resolution and repair ensues by Mϕ recruitment (35). In this phase there is a switch from pro-inflammatory prostaglandins to anti-inflammatory lipoxins, which inhibit the recruitment of PMNs and promote monocyte recruitment (35). Resolvins (36) and protectins (37) produced by Mϕ aid in resolution.

When tissue is damaged, ATP, potassium (K^+)-ions, uric acid, high mobility group box domain-1 (HMGB1), and members of the S100 calcium (Ca^{++})-binding protein family are released (38). These substances can induce several pathways. ATP binds with Mϕ surfaces causing an efflux of K^+-ions which activate NALP3 inflammasome (39). HMGB1 and the S100

Ca^{++}-binding protein family act with the receptor for advanced glycation end products (RAGE), which cooperates with TLRs to induce the inflammatory response (40, 41).

Damage to the endothelium results in leakage of plasma proteins and platelets into extravascular spaces; one of these plasma proteins is Hageman factor/factor XII which activates upon contact with the ECM. This generates four cascades: the kallikrein-kinin cascade, the coagulation cascade, the fibrinolytic cascade, and the complement cascade, leading to inflammation (Chapter 1, Figure 3).

Endogenous inducers often found in joints are crystals of monosodium urate, Ca^{++} phosphate dehydrate, advanced glycation end products (AGE) and oxidized lipoproteins. Mϕ recognize these inducers and treat them as foreign bodies, triggering inflammasomes, caspases, and IL-1 family members (42, 43).

The inflammasome is a large complex that controls the activation of caspase-1 (44, 45). Caspase-1 regulates IL-1β, IL-18, and pyroptosis/inflammatory cell death (46). Once caspase-1 is recruited into the inflammasome there is self-induced cleavage of pro-caspase-1 containing caspase activation and recruitement domain (CARD), which further helps the assembly of the inflammasome (47). Caspase-11 has also been linked to signaling of the inflammasome (44), helping to mediate processing of IL-1β and IL-18. Apoptosis speck-like adaptor protein containing a CARD (ASC) plays a central role between NLR and caspase-1 in the inflammasome (48). There are several types of inflammasomes, these include: NLRP3 inflammasome, NLRC4 inflammasome, NLRP1 inflammasome, NLRP6 inflammasome, NLRP12 inflammasome, and pyrin domain and HIN domain/AIM2 inflammasome. The NLRP3 inflammasome is the most widely studied and is activated by various pathogens, endogenous signals, as well as environmental signals (46). Three mechanisms have been proposed for NLRP3 activation: mitochondrial ROS, potassium efflux, and destabilization of phagolysosomes after digestions of

certain particulates (49). The NLRC4 inflammasome responds to bacterial flagellin and prgJ (50), and the NLRP1 inflammasome is activated by *bacillus anthracis* (51). NLRP6 and NLRP12 have been linked to the function of the inflammasome to induce caspase-1 processing of IL-1β (52, 53). The AIM2 inflammasome responds to both viral and bacterial infections (54). Interferons have been shown to play an important role in regulating of inflammasomes. They not only respond to infections, but anatagonize IL-1β production by decreasing the amount of intracellular pro-IL-1β and by inhibiting caspase-1 activation (55). Interferon induces IL-10 which inhibits pro-IL-1β and pro-IL-1α via the STAT3 pathway. Various other cytokines and chemokines have also been implicated in influencing inflammasome signaling (56). The inflammasome particularly NLRP3 has been shown to play a central role in wound healing and fibrosis. ASC is secreted by myofibroblasts (57), and NLRP3 inflammasomes can drive fibrosis (58-60). See chapter 1 figure 4 for a schematic of the inflammasome.

Another group of endogenous inducers are the breakdown products of the ECM which activate TLR4 and promote tissue repair (61). This list of known endogenous inducers is growing, and more research is being done to understand their role in inflammation.

2.1.2. Mediators

Inducers of inflammation trigger the production of inflammatory mediators which can be derived from plasma proteins or secreted from cells. Inflammatory mediators can be organized into seven groups: vasoactive amines, vasoactive peptides, complement components, lipid mediators, chemokine, cytokines, and proteolytic enzymes.

Vasoactive amines (histamine and serotonin) are produced upon degranulation of MCs and platelets (62). They increase vascular permeability and either vasodilation or vasoconstriction.

Vasoactive peptides (substance P, kinins, fibrinopeptide A/B, and fibrin products) can be stored in secretory vesicles in an active form or generated by proteolytic modification of inactive precursors in the ECM.

The complement components are C3a, C4a, and C5a; they trigger granulocyte and monocyte recruitment affecting inflammation and the vasculature.

Lipid mediators (eicosanoids, and platelet activating factors (PAF)) are derived from phospholipids via the arachadonic acid pathway (Chapter 1, Figure 5). Lipoxins and eicosanoids of omega-3 fatty acid derived resolvins and protectins inhibit inflammation and promote resolution and tissue repair (63). PAFs promote inflammation by aiding in leukocyte recruitment, increasing vascular permeability, and promoting vasodilation or vasoconstriction (64).

Chemokines are produced by a variety of cells in response to inducers of inflammation. Their main role is in chemotaxis and leukocyte recruitment. Inflammatory cytokines are also produced by a variety of cells; they activate the endothelium, activate leukocytes, induce acute-phase reactants, and promote further release of cytokines. Proteolytic enzymes (elastin, cathepsins, and matrix metalloproteinases (MMP)) degrade the ECM and basement membrane promoting leukocyte migration and tissue remodelling.

2.1.3. Resolution

After initiation and mediation of the inflammatory response, resolution ensues. In some cases the inflammation resolves and normal tissue architecture and physiology are restored (as in acute inflammation) in other cases the inflammatory reaction persists leading to tissue damage, scar formation, and fibrosis (chronic inflammation).

2.2. Acute inflammation

Acute inflammation includes: accumulation of plasma and fluid, intravascular stimulation of platelets, and the presence of PMNs. It is the immediate response to injury or infection. The

vasculature plays a major role in acute inflammation by 1) an increase in blood flow, 2) structural changes allowing plasma proteins and leukocytes out of circulation, and 3) transmigration of leukocytes into the target tissue.

2.2.1. Vascular changes

Changes in vascular flow and calibre occur early after injury and are critical for the development of the acute inflammatory response. These pathological changes were first characterized by Sir Thomas Lewis as the “triple response”, in experiments showing flare and wheal in skin trauma (65, 66). The triple response occurs as follows:

1) Transient vasoconstriction of arterioles occurs at the injured site. This chemical and neurogenic mediated event lasts only seconds.
2) Vasodilation occurs, resulting in the development of new capillary beds increasing blood flow. This causes heat and redness and a condition known as hyperemia. This is followed by the slowing of circulation.
3) Increased permeability of microvasculature causes edema, leakage of fluid into the extravascular space. The loss of intravascular fluid causes an increase in blood viscosity, and stasis. As stasis progresses, leukocytes (mainly PMNs) orient themselves peripherally along the vascular endothelium and leukocyte migration (also known as emigration or transmigration) occurs.

Increased permeability leads to protein-rich fluid leaking into the extravascular tissue. This loss of protein from the plasma causes an increase in hydrostatic pressure within the vessels causing more fluids to flow out and build up in the interstitial tissue (edema). There are several possible mechanisms by which the endothelium becomes leaky. The first is by direct injury to endothelial cells, often from burns (67) or infections, causing endothelial necrosis (68). Leakage

is immediate after injury and lasts for hours until the vessels are repaired. The second is injury mediated by leukocytes which adhere to the endothelium. Leukocytes can release ROS and proteolytic enzymes after adherence to the endothelium, which causes endothelial injury (69). The third mechanism is that there is increased permeability of the endothelium through the formation of gaps in the venules after exposure to histamines, bradykinins, substance P, and leukotrienes (70-74). This is rapid and short lived. The fourth is through cytoskeletal reorganization induced by a variety of cytokines, hypoxia or stress signals. The normal, resting endothelium has canals in it (75); the fifth mechanism is excessive leakage out of these endothelial canals after exposure to toxins. Knowing whether the increased permeability of the vasculature is caused by direct or indirect injury to the endothelium is of the utmost importance clinically, in order to use proper therapeutics.

There are many vasoactive mediators that increase permeability; these include phospholipid metabolites and arachadonic acid metabolites (Chapter 1, Figure 5), platelets and their granules, MC and basophil products, factors released from the endothelium itself, cytokines released by Mϕ, PAF, Haegman factor, kinins, and complement.

2.2.2. Cellular events

A key function of inflammation is the delivery of leukocytes to the injured site. Leukocyte recruitment occurs within the postcapillary venules, starting with rolling and adherence. After blood flow has slowed and leukocytes appear at the periphery, selectins of the endothelium and integrins on the leukocyte and endothelium are presented (32). These molecules tether the leukocytes, slow them down, and eventually stop them. After stopping, the leukocytes migrate through the endothelium and undergo chemotaxis to get to the site of injury. In acute inflammation this process is dominated by PMNs during the first 6-24 hours and by monocytes/ Mϕ in the next 24-48 hours. Chemotaxis/extravasation of leukocytes occurs in response to either

exogenous agents (bacterial products) or endogenous agents (complement, cytokines, and lipoxigenase) (32).

When PMNs and Mϕ arrive and accumulate in the tissue, they phagocytose debris and release enzymes in order to kill pathogens, remove damaged tissue, and other foreign particulates. This process was first described by Eli Metchnikoff as three distinct phases: 1) recognition and attachment of particles by leukocytes, 2) engulfment and phagocytic vacuole formation, and 3) killing/degradation of ingested material (76).

Opsonins coat microorganisms which bind to specific receptors on leukocytes. The main opsonins are: the Fc fragment of IgG, the C3b fragment of complement, and collectins (77). The corresponding leukocyte receptor is FcγR, which recognizes the Fc fragment of IgG and the complement receptors. After the opsonized particle binds to FcγR, the cell extends pseudopods which are closely applied to the particle; they fuse around the particle resulting in a phagosome. The phagocytic vacuole (phagosome) membrane fuses with the membrane of a lysosomal granule (PMN degranulation) causing the granule's contents to be released into the phagolysosome (phagocytic lysosome) (78, 79). When the phagosome does not close completely, granule contents can be released out of the cell, which may cause further damage. Once endocytosis occurs, the phagocyte can no longer ingest particles. The last step in phagocytosis is the killing/degradation of particles inside the phagolysosome. This process is oxygen-dependent. PMNs are stimulated by the phagocytotic process to increase oxygen consumption. Oxygen consumption generates metabolites (superoxide, hydrogen peroxide, hydroxyl radicals, and hydrochlorous acid) by activation of NADPH oxidase (80). Hydrogen peroxide is a potent bacterial killer.

Bacterial killing can also occur via non-oxidative mechanisms. These include bactericidal permeability increasing protein (BPI), lysosomal hydrolases, defensins, lactoferrins, lysozymes,

and major basic proteins/bactericide from eosinophils. After the bacteria have been killed, acid hydrolases in the PMN azurophilic granules degrade the bacteria with the phagolysosome (pH 4-5) (81).

2.2.3. Tissue injury by leukocytes

As just described, leukocytes are capable of combating foreign particles; they are also capable of damaging host tissue. PMNs release important substances into the ECM that induce tissue damage (82). These include:

1) Lysosomal enzymes released from PMN granules. These enzymes are beneficial when active intracellularly, but are harmful when released into the ECM (83).
2) Activated oxygen species (NO•) can be produced in injured tissue and can damage both tissues and cells (84).
3) Arachadonic acid metabolites (prostaglandins and leukotrienes) promote further inflammation.

After phagocytosis, PMNs undergo rapid apoptosis and are ingested by Mϕ (85), which is dependent on integrin Mac-1/CD11b on PMN surfaces. This plays a very important role in acute inflammation.

2.2.4. Acute inflammatory outcomes

In response to acute inflammation, there can be one of four outcomes. The first is complete resolution. This is the most common outcome after injury when there is mild tissue damage; it is largely through PMN apoptosis. The second is abscess formation. This occurs most commonly after an infection. The third is healing by connective tissue replacement/fibrosis. This occurs when the inflammatory response is within tissue that does not regenerate or when there is a massive amount of fibrin. The fourth and final possible outcome of acute inflammation is the

progression to chronic inflammation. This may occur almost immediately after the onset of acute inflammation or may occur upon failure of acute inflammation to resolve.

2.3. Chronic inflammation

Chronic inflammation is much less well understood than acute inflammation. It may be considered a sequel to acute inflammation, or an immune response to a foreign antigen, or an autoimmune response. Histologically, chronic inflammation is characterized by: infiltration of mononuclear cells, tissue destruction, angiogenesis, and fibrosis. The cellular components of chronic inflammation are monocytes/Mϕ, lymphocytes, MCs and PMNs, and eosinophils.

Mϕ arise from monocytes made in the hematopoetic blood marrow; when monocytes leave circulation and enter various tissues they differentiate into Mϕ. Mϕ can live for several months within tissues. As discussed in acute inflammation, monocytes appear within 24-48 hours after the initiation of inflammation. Upon their arrival at the target tissue, they transform into Mϕ, which are activated resulting in their ability to kill foreign particles, to generate inflammatory mediators (IL-1, TNF), and to regulate lymphocyte response. If these processes are unregulated, tissue injury and fibrosis characteristic of chronic inflammation ensue (86). In chronic inflammation the persistence of Mϕ can be due to continued monocyte recruitment, local proliferation of Mϕ, or immobilization of Mϕ by Mϕ inhibitory factor (MIF) and lipids (87). Macrophages are critical in the chronic inflammatory response because of their release of numerous factors that are toxic to cells and the ECM.

Lymphocytes (T or B) are part of both the antibody-mediated and cell-mediated immune responses as well as non-immune mediated inflammation. Lymphocytes and Mϕ have complementary responses; Mϕ regulate lymphocyte responses to antigens and activation by cytokine release, and lymphocytes produce lymphokines (IFNγ) that stimulate monocytes and Mϕ (88). T lymphocytes also modulate production of antibodies and modulate cell cytotoxicity.

MCs are found in both acute and chronic inflammation. MCs act in chronic inflammation by increasing cytokines thereby attracting leukocytes to the site of injury. PMNs are classically thought to be part of the acute response, but can also be observed in tissues of chronic inflammation. PMNs are induced by the up-regulation of chemokine receptor (monocyte chemoattractant protein 1; MCP1) in chronic inflammation (89).

2.4. Interleukin 1

Interleukin 1 is a pro-inflammatory molecule first described in the 1940's as an endogenous pyrogen (90). IL-1 was later discovered to have many inflammatory properties which were mediated by two similar cytokines which bind to the same receptor: IL-1α and IL-1β (91, 92). The genes encoding for IL-1α and β are on chromosome 2 (93). IL-1α and β form a three dimensional structure of a β-trefoil fold formed by 12 β-strands (94). Both cytokines are produced by monocytes and macrophages and to a lesser degree neutrophils, keratinocytes, epithelial cells, endothelial cells, lymphocytes, smooth muscle cells and fibroblasts (95). IL-1α and β are synthesized as precursors (31kD) which are cleaved into their mature forms (17kD) and secreted independent of the golgi apparatus and endoplasmic reticulum unlike other cytokines (96).

Pro-IL-1α is a membrane associated protein involved in paracrine cell-cell signalling (97). Pro-IL-1α can exert intracellular effects but requires N-terminal nuclear localization sequence (98). IL-1α is normally not detected in the blood unless there is severe illness, where

the cytokine is coming from dying cells. In the past few years the release of IL-1α has been discovered to be broader than just that from dying cells. After TLR activation IL-1α is expressed and localized to the cell surface as an active membrane bound ligand (99). There is some evidence suggesting that NLRP3 inflammasomes may have a direct role in the regulation of IL-1α (99, 100). After IL-1α activation by the inflammasome IL-1α is actively secreted. It has been proposed that IL-1α binds IL-1β and is co-secreted (99). Yazdi and Drexler (101) have proposed two other pathways of IL-1α cleavage and secretion: caspase-1 dependent and independent pathways. With either pathway IL-1α processing is mediated by calcium dependent calpain-like proteases, which are downstream to the inflammasome. Despite a large body of research on inflammation evidence for the role of IL-1α in inflammatory disorders in limited; however, IL-1α seems to be important for inflammation (99, 100).

Unlike pro-IL-1α, pro-IL-1β is biologically inactive and therefore must become the functional mature IL-1β (102). Pro-IL-1β is cleaved by caspase-1 (103), which is activated by the NALP3 inflammasome. In order for IL-1β release from Mϕ there must be activation by TLRs causing transcription and translation of pro-IL-1β, as well as a NLP process in which IL-1β is processed and released. This process is mediated by caspase-1 mechanism involving P2X purinoceptor 7 activation by ATP (104). Pro-IL-1β can also be cleaved extracellularly (105).NFκB inhibits caspase-1 preventing the release of IL-1β by Mϕ (106).

Both IL-1α and IL-1β stimulate local and systemic inflammatory responses that can lead to both acute and chronic inflammatory diseases. IL-1s produce phospholipase A2, COX2, and inducible nitric oxide synthase causing inflammatory nitric oxide and prostaglandin release. IL-1s also stimulate MMPs causing breakdown of articular cartilage (96) and indirectly stimulate bone erosion via osteoclast maturation (98).

3. GROWTH FACTORS

Growth factors are tightly linked with inflammation in the knee. There are several growth factors which are active in the knee joint. TGFβ is a member of a family of polypeptide growth factors which include activins, inhibins, bone morphogenic proteins, and various differentiation factors. The TGFβ superfamily contains TGFβ as well as mullerian inhibitory substance and glial cell line-derived neurotrophic factor (107). TGFβ is a dimeric molecule comprised of two monomers, a β-strand and an α-helix. TGFβ is important in connective tissue proliferation, and contributes to both the influx of inflammatory cells as well as the activation of fibroblasts to differentiate into myofibroblasts (108). There are three isoforms of TGFβ (1,2, and 3); TGFβ1 is the predominant isoform in the immune system and is important in immunoregulation (109, 110). T cells are involved in severe inflammation, and therefore will not be discussed in this thesis. TGFβ is synthesized in an inactive form as a dimeric pre-pro-TGFβ precursor, which then forms a complex which is secreted from cells bound to matrix proteins (111). In order to free the active TGFβ proteolysis, cell surface proteins and a low pH are necessary. TGFβ signalling binds to TGFβ receptor II recruiting type I receptors which activate Smad transcription factors (Smad2, Smad3, and Smad4) (112). Smad2 /3 are phosphorylated and activated by TGFβ receptor and heterodimerizes with Smad4 into a Smad complex, which transclocates into a nucleus to regulate transcription or target genes. TGFβ also works via other mechanisms, including activation of the Ras-extracellular signal regulated-kinase, TGFβ-activated kinase-mitogen-activated protein kinase kinase 4c-Jun N-terminal kinase, TAK1-MKK3/6-p38, Rho-Rac-cdc42 mitogen-activated prontein kinase, and phosphatidylinositol 3-kinase-Akt pathways.

Cytokines act in signalling TGFβ; in particular IL-6. There is cross-talk between IL-6 and TGFβ, which has been identified within various tissues (113-116) and shown to inhibit IL-8 (117). IL-6 enhances TGFβ receptor internalization and thereby enhances TGFβ signalling (118).

IL-13 is also involved in TGFβ signalling, inducing the expression of TGFβ in Mϕ (119). This process is in two stages involving induction of IL-13Rα^2 via TNFα and IL-13 signalling via IL-13Rα^2 activation of TGFβ promoter. TGFβ causes the downregulation of all MMPs except MMP13. TGFβ has different effects in different joint tissues; it is critical for cartilage development and changes in TGFβ result in terminal differentiation of chondrocytes and changes in the cartilage matrix (120). Whereas, in ligaments and synovium it both helps in healing as well as promotes fibrosis in chronic inflammation by fibroblast proliferation and collagen synthesis. BMP, a member of the TGFβ family is critical for chondrocyte growth as well as bone formation. Due to these different effects in tissue TGFβ has multiple roles in the development of PTOA: causing scar formation of the ligaments, synovium and meniscus, changing cartilage homeostasis, and causing subchondral bone changes and osteophyte formation.

FGF are heparin-binding proteins involved in angiogenesis, embryonic development and wound healing. The FGF family includes FGF1-FGF10; FGF2/bFGF is critical in wound healing. FGF ligands are signalled via cell surface FGF receptors (FGFR1-FGFR4) Tyr kinase. After ligand binding FGF receptors dimerize causing intracellular receptor kinase domains to reorient for transphosphorylation and activation of receptor kinases (121). The activated receptor kinases phosphorylate and activate FGF substrate 2α and phospholipase Cγ1 (122, 123). This initiates signalling through the Ras-mitogen activated protein kinase pathway or phosphatidylinositol 3-kinase-Akt pathway, causes intracellular calcium release, and activates protein kinase C (122, 123).

All FGFs interact with heparin sulphate glycosaminoglycan chains of heparin sulphate proteoglycans present in the extracellular matrix (124). bFGF is produced by cartilage and sequestered there by perlecan (125), which when injured releases bound bFGF activating the

extracellular signal regulated-kinase pathway. bFGF appears to play a key role in cartilage homeostasis; however, its exact role is controversial.

4. OSTEOARTHRITIS

OA is one of the main causes of pain, disability and functional loss in North America. By 65 years of age, well over 50% of people have some radiographic evidence of OA and by 75 years of age, some 80% of the population has positive radiographic findings of OA. While OA affects multiple joints, the knee is the most common, affecting ~1% of adults age 25-34 years and increasing with age (126). There are two types of OA: primary OA and secondary OA. Primary OA arises spontaneously from an unknown cause, whereas secondary OA results from a known cause, for example, an infection or a previous injury.

Osteoarthritis (OA) is characterized by hyaline articular cartilage degradation and wear and chronic joint pain that is associated with recurrent attacks of more acute synovial inflammation superimposed on a background of lower grade chronic synovial inflammation. As the cartilage wears away, the bone surfaces become less protected by the cartilaginous cover (127). At that end stage of disease, nerve endings in the bone are left exposed, causing patients to experience severe pain when doing any load bearing activities, including simply walking. Due to the pain, patients often reduce their activity which can lead to a serious reduction in their quality of life. OA is thus defined by these symptoms and by radiographic changes with effusions, osteophytes, bone sclerosis, subchondral bone cysts and the increasing loss of joint space (as the cartilage wears away).

4.1. Diagnosis and epidemiology

Clinically, OA is graded using a standard scheme on plain X-ray films known as Kellgren-Lawrence (KL) grading. As noted above, the features that are graded are joint space narrowing, bony contour abnormalities, subchondral sclerosis, osteophytes, and cyst formation (128). The current and most widely used criteria for diagnosis OA correlate these radiographic grades with knee pain for most days in the prior month, osteophytes, synovial fluid changes,

morning stiffness and crepitus (129). Routine histological diagnosis is not performed clinically; however, the microscopic features of OA in the articular cartilage consist of chondrocyte and proteoglycan loss, cartilage ulceration and erosion, subchondral degeneration, and osteophytosis (130). Histological diagnosis is the gold standard, used to quantify OA in animal models (130).

4.2. Systemic risk factors

Several risk factors have been associated with the development and progression of OA. Dieppe developed a model of OA pathogenesis to separate risk factors into systemic factors leading to overall disease risk and mechanical risk factors (131).

The most compelling systemic risk factor links OA with age (132, 133), most notably in the knee and hand (126, 134). Males and females have comparable OA risk throughout reproductive life (126); however, post-menopausal women have an increased predisposition for disease. Hormone replacement therapy (HRT) is protective of osteophytes and joint space narrowing, but protection fails to continue once HRT is discontinued (135). Women using estrogen replacement therapy also have larger tibial cartilage volumes compared to women that had never taking HRT (136). Obesity is the second strongest risk factor for knee OA (137). Many studies have shown a strong relationship between body mass index and knee OA (138, 139). This likely is not unique to the knee (140) suggesting this association is not simply mechanical but may be due to metabolic changes or inflammation. Other risk factors include bone density (141), genetics (142), and nutritional status (143).

4.3. Mechanical risk factors

Overall risk of secondary OA development is based on systemic risk factors, but mechanical factors are linked with specific sites affected. It is thought that there is a type of injury-induced, mechanical abnormality of the injured joint and a subsequent interplay between the altered mechanics, biological changes (inflammation and cartilage damage) and the development of OA.

It is widely accepted that joint injury is a risk factor for knee OA (144-146). While injury to any structures in the knee can lead to OA, ACL injuries are the most likely to cause severe and rapid OA of the joint (147). These injuries result in gait abnormalities leading to increased joint loading (148). Acute surgical intervention is recommended for most ACL injuries (149-151); however as of 2013, surgical reconstruction has not been shown to retard the progression of OA (1, 152, 153).

Inflammation appears to be playing a role in all types of OA, based on preliminary laboratory data examining up- and down-regulation of inflammatory factors (154). Inflammation is a normal part of tissue healing/repair (95, 155). After an injury, damaged tissue and cells send out a variety of signals in order to start the repair process. Traditionally OA was seen as an articular cartilage disease; however, more recently OA has been viewed as a complete joint organ disease and an inflammatory disorder (156), and therefore must be studied as such.

In secondary OA (eg after a joint injury) it is expected that inflammation would begin acutely in response to the injury (a wound healing response), fail to resolve, and the joint would thus become chronically inflamed. After the onset of OA (secondary or primary), the innate inflammatory pathways appear to be constantly stimulated. Scanzello and colleagues (157) have suggested that OA, in essence, is a “chronic wound”.

4.4. Wound healing

Wound healing consists of three phases: an inflammatory phase, a proliferative phase and a remodelling phase. The inflammatory phase begins immediately after injury starting with hemostasis in which the wound fills with blood; the exposure of blood causes platelet degranulation and activation of the Hageman factor/factor XII (Chapter 1, Figure 3). Platelets secrete inflammatory factors and express glycoproteins causing the formation of an aggregate. A clot is formed and the injury signals are amplified attracting chemokines to the wound site.

Prostaglandins lead to vasodilation resulting in edema at the site of the injury. Circulating immune cells start to appear in the wound followed by leukocytes, which release pro-inflammatory cytokines leading to propagation and tissue formation (proliferative phase; see Chapter 1 Figure 6 for cascade).

The onset of the proliferative phase is marked by an infiltration of fibroblasts at the wound site. In this phase angiogenesis occurs, collagen matrix is laid down, as well as other components of the ECM, and fibroblasts differentiate into myofibroblasts contracting the wound. These events signal the remodelling phase of wound healing in which collagen production and degradation equalize, fibers are rearranged and cross-linked longitudinally, and any remaining structures that are no longer needed undergo apoptosis.

While bone injury and healing follow a similar process to soft tissue wound healing, there are other factors that come into play, such as the influence of multipotent hemopoietic stem cells, matrix and matrix fragments, and mechanical load changes to the bone matrix influencing joint mechanics. This may increase the risk of the development of OA after injury or surgery in the knee joint.

4.5. Anterior Cruciate Ligament injury and reconstruction

As discussed above, the ACL is important in maintaining joint stability during motion. After an ACL injury, there are significant changes in tibiofemoral motion during gait with more internal tibial rotation and posterior tibial translation during stance phase (158), impingement of the medial tibial spine by the femur during lunge activities (159). These mechanical abnormalities result in a shift in joint loading leading to altered tension and compression of the cartilage resulting in changes in cartilage integrity and increased catabolic activity ultimately causing OA (reviewed in (22)). Therefore, in theory OA should not develop if mechanical changes could be prevented or minimized. Many studies have investigated the potential role that

reconstruction plays in preventing joint degeneration; however, it appears that the increase in stability acquired from surgery plays only a limited role in preventing and retarding the progression of OA (23, 160-162). Evidence from our lab has indicated that some types of surgery may even be involved in provoking the development of OA. Therefore some inherent change at the time of injury or during wound repair must be leading to the development of OA; one possibility is inflammatory changes in the joint.

5. DETAILS OF INFLAMMATION AND OSTEOARTHRITIS

As noted above, inflammation is a normal part of tissue healing/repair (95, 155). After an injury, damaged tissue and cells send out a variety of signals in order to start the repair process. While OA traditionally was not seen as an inflammatory disorder, reports over the past 15 years have linked inflammation and OA (156).

Cytokines are proteins produced by various cells that modulate the function of other cells. They play important roles in both acute and chronic inflammation, and by their inhibition both can be modified. Cytokines can regulate lymphocyte function, activation, and differentiation. Some cytokines can stimulate hematopoeisis (IL3, IL7, and colony stimulating factors). Other cytokines are involved in natural immunity (IL-1β, TNFα, IFNα/β, and IL-6) and activate inflammatory cells in both acute and chronic inflammation (IFNγ, TNFα/β, IL5, IL-10, and IL-12). Several major cytokines are thought to be involved in OA; these include interleukins: L-1β, IL-1α, IL4, IL-6, IL-8, IL-10, IL-11, IL-13, IL7, LIF (leukemia inhibitory factor), TNFα (tumor necrosis factor-α), and IL-1Ra (IL-1 receptor antagonist). Several growth factors are also involved, for example TGFβ, FGF, PDGF, and IGF. Some of these are up-regulated such as IL-10 and TGFβ (negative regulators of lymphocytes); while others such as IL-1 and IL4 are down-regulated (promote lymphocyte growth). The cytokines involved can be divided into pro-inflammatory and anti-inflammatory types.

5.1. Pro-inflammatory agents

Pro-inflammatory cytokines are thought to be pivotal in the initiation and development of OA, with IL-1β, and TNFα in the forefront. IL-1β is produced by macrophages and endothelial cells in the synovial membrane and cartilage-pannus junction (163). IL-1β is elevated in rheumatoid arthritis (RA) (164, 165) and in OA. It is activated through cell-surface receptor binding (IL-1RI and IL-1RII). IL-1RI is found in articular tissue cells and appears to be

responsible for signal transduction (Chapter 1, Figure 7). The number of IL-1RI is significantly higher in OA chondrocytes and synovial fibroblasts (166-168). While IL-1RII, which lacks a cytoplasmic domain, cannot transmit a signal, it appears to act as a decoy receptor, inhibiting IL-1β (169). IL-1RII is elevated in patients with RA (170), and has been seen as a possible therapeutic avenue.

TNFα also appears to be an important mediator of synovial inflammation and matrix degradation in OA. TNFα is elevated in RA and in OA, with increased production by synoviocytes, leukocytes and chondrocytes (171). Activation occurs through signal transduction via binding to the membrane bound TNF-Rs; TNF-R expression in OA chondrocytes and synovial fibroblasts is elevated (172, 173).

IL-1β and TNFα may be the most relevant cytokines in OA; however, not all catabolic activity in OA can be attributed to these two. IL-1β is highly up-regulated in the synovium and synovial fluid of patients with OA, and is a potent inducer of inflammation. Certain IL-1 haplotypes have been associated with knee OA (174) and its severity (175). Other pro-inflammatory cytokines include: IL-8, a chemotatic cytokine (176), LIF, IL-1, IL-6, an IL-1 inducer (177), and IL-17, involved in IL-1β and TNFα up-regulation (178).

5.2. Anti-inflammatory agents and antagonists

It is believed that cartilage degradation in OA is the result of a disruption in the homeostasis of catabolic cytokines, anabolic cytokines and anti-inflammatory cytokines/antagonists. Three anti-inflammatory cytokines are up-regulated in synovial fluid of OA patients: IL4, IL-10 and IL-13. They decrease the production of IL-1β, TNFα, and MMPs (matrix metalloproteinase), up-regulate IL-1Ra and TIMP-1 (tissue inhibitory metalloproteinase), and inhibit PGE2 (prostaglandin E2) release (179-182).

IL-1Ra is secreted from monocytes, macrophages and neutrophils. Three isoforms have been identified: IL-1sRa (soluble and extracellular), icIL-1Ra1 (intracellular), and icIL-1RaII (183). IL-1Ra competitively binds to IL-1RI; it does not induce any cellular response. IL-1Ra binds to IL-1RI inhibiting IL-1β activity (Chapter 1, Figure 7). It blocks many pathologies seen in OA, including PGE2 synthesis (184), collagenase production, and matrix degradation. IL-1Ra concentrations are significantly higher in the synovial fluid of RA patients (185-187) and OA patients (186). IL-1Ra knockout mice spontaneously develop inflammatory arthritis (188), suggesting IL-1Ra is a critical player in inflammation.

IFNβ (interferon β) also has anti-inflammatory effects such as decreased expression of IL-1 and TNFα and increased expression of IL-1Ra. INFβ increases the production of IL-1Ra by a variety of cell types (189-191), including synoviocytes and other cells residing in the joint (192).

5.3. IL-1β and IL-1Ra balance

The balance between IL-1Ra and IL-1β levels influences the physiological and pathological effects in various tissues. IL-1Ra must be produced in abundance in local tissues to effectively block the effects of IL-1β (193-197). This likely explains why, despite increased levels of IL-1Ra found in OA, joints exhibit OA pathologies. Many studies have shown an imbalance in the ratio of IL-1β: IL-1Ra in RA (198, 199) and in chondrocytes of OA (200).

6. TREATMENTS FOR OSTEOARTHRITIS

There are a variety of treatment strategies targeted at OA; but as of 2013, currently none of them are curative. Management strategies vary from lifestyle modification to pharmacological to surgical. Weight loss of ~20% body weight is the only intervention which has been shown to slow the progression of OA (201-203). Unfortunately this is extremely difficult for most patients and in the cases of post-traumatic OA patients often have a normal BMI. Pharmacological treatments include pain management aids and disease modifying drugs. Analgesics such as acetaminophen and NSAIDs are widely used; NSAIDs tend to be more efficacious but have a greater side effect profile (204). Selective NSAIDs have been used; however, there are cardiovascular concerns when using COX2 inhibitors. If the patient fails these treatments or they are contraindicated, opioids can be used for pain management, but their addictive nature and adverse effects make them a less desirable option. Some newer pain management strategies employ the use of serotonin reuptake inhibitors, Duloxetine being the most widely studied. Duloxetine improves pain scores and WOMAC scores compared to placebos (205-208), and is recommended by the American College of Rheumatology as a treatment for patients who have had an inadequate response to conventional treatments (209). Alternative or complementary medicines for the treatment of OA are also widely studied; these are controversial and will not be discussed in this thesis.

Several intra-articular injections are widely used to manage OA. Corticosteroid injections are also widely used and found to reduce pain for up to 2 weeks post-injection (210). A more detailed discussion on corticosteroids is presented below. Other intra-articular therapies include bone morphogenic protein-7 (BMP7), fibroblast growth factor-18 (FGF18), botulinum toxin A (Botox), platelet rich plasma (PRP), mesenchymal stem cells (MSCs), and hyaluronic acid. BMP7 decreases cartilage degradation in various animal models (211) and has passed phase I

clinical trials (212). Intra-articular FGF18 injections in animal models of OA have increased cartilage repair and chondrogenesis (213). There is some potential for the use of Botox for refractory OA. Because of its nocioceptive properties pain scores are improved with Botox injections (214-218). Autologous PRP has been used in various musculoskeletal injuries for the past 10-15 years (reviewed in (219), and has recently been considered for use in OA. However, clinical studies have failed to show any differences with PRP (220-224). Despite minimal improvements in pain scores none of the studies showed cartilage improvement. The use of MSCs in many disorders including OA is a current popular research area and studies show varied results in animals. Two case studies have shown some significant differences in outcomes with treatment (225, 226). Hyaluronic acid is widely used clinically and many patients claim symptom relief; however, recent studies indicate hyaluronic acid has little or no effect on OA (227).

Several biological agents have been investigated in animal models and have recently been studied in clinical trials. IL-1Ra has demonstrated potential in both animal models and clinical trials; these will be discussed below. TNFα antagonist is used as a second or third line treatment in RA, and several trials have evaluated its use in OA. These trials, done in erosive hand OA, have shown a lack of efficacy in preventing or reducing any primary or secondary outcomes (228, 229). One of the newest treatments being explored is tanezumab, an anti-nerve growth factor. Several trials have shown improved WOMAC scores and pain assessment scores with treatment (230-233). Note that of all the treatments discussed none has been shown to modify the progression of OA, rather they have helped manage the symptoms. A drug normally used to treat osteoporosis, strontium ranelate, has been shown to change joint space narrowing (234), and may be the first disease modifying drug to date.

Often as a later treatment surgical intervention is offered to patients; the choice of surgical treatment depends on symptoms, disability, radiographic change, and patient age. Options consist of debridement, high tibial osteotomy in the young, and arthroplasty. Debridement has the same results as the current physical and medical therapies used (235). Joint arthroplasty is generally reserved for patients with severe disease, persistent pain, functional limitations, and reduced quality of life. 80% of patients report pain reduction after surgery in prospective studies. High tibial osteotomies can be an option in younger patients with unicompartmental disease to delay the need for arthroplasty (236).

6.1. Glucocorticoids

Corticosteroids used to intervene in inflammation are glucocorticoids (cortisol, prednisolone, dexamethasone, betamethasone, rimexolone, triamcinolone) that control carbohydrate, fat and protein metabolism, and act as anti-inflammatory agents regardless of the cause of inflammation. Glucocorticoids work via a variety of mechanisms as a result of stress response via the sympathetic nervous system (237), activating the release of norepinephrine which enhances anti-inflammatory mediators (IL-10, TGFβ) and inhibits pro-inflammatory cytokines (IL-12, IFNγ, TNFα) (238). In response to stress the hypothalamic-pituitary-adrenal axis and hypothalamic-autonomic nervous system axis are activated. These pathways are part of the endocrine and neurological systems which supress immune, inflammatory and nervous responses (239). The hypothalamus releases corticotropin releasing hormone and arginine-vasopressin which acts on the anterior pituitary causing the release of adrenocorticotropin hormone (ACTH) (240). ACTH acts on the adrenals stimulating the release of cortisol, which in turn inhibit corticotropin releasing hormone and ACTH (241). Cortisol released from the adrenals enters the blood stream either unbound which exerts systemic effects or bound to cortisol-binding globulin. The hypothalamic-autonomic nervous system causes the release of

norepinephrine which stimulates IL-10 and TGFβ and inhibits IL-12, TNFα, and interferon (242). The sympathetic nervous system and adrenal axes act on each other with stimulatory effects of corticotrophin releasing hormone and norepinephrine on each other (243).

Glucocorticoids enter the cell via passive diffusion and bind to intracellular glucocorticoid receptors. Glucocorticoid receptors are part of the ligand regulated nuclear receptor superfamily found in the cytosol transporting into the nucleus upon ligand-receptor binding (244). Several molecules (hsp90, hsp70, hsp56, p23, p50 and CYP40) are associated with glucocorticoid receptors called the foldosome (245) in order for proper folding and steroid binding (246). The foldosome complex is bound in the cytoplasm to two receptor isoforms: GRα and GRβ (247). Ligands can only bind to GRα and GRβ binds to the glucocorticoid response element promoter sequence. Membrane bound receptors have also been identified in the cell membranes of immune cells (248), and have been found upregulated in certain diseases (249, 250). Glucocorticoid receptor N-terminal activation function domain contains serine residues which are phosphorylated by mitogen activated protein kinase (MAPK) and cyclin dependent kinase (251). Phosphorylation of the receptor acts on nuclear translocation, nuclear export, transcription, and receptor stability (252).

After the glucocorticoid-GRα complex is translocated into the nucleus in homodimerizes with another glucocorticoid-GRα complex which then binds to the glucocorticoid response element and causes gene transcription (253). Glucocorticoids bound to receptors can also directly bind to a transcription factor without dimerization leading to inhibition of transcription and of the receptor (254, 255). This is the mechanism by which glucocorticoids primarily exert their anti-inflammatory effects.

Glucocorticoids can also act non-genomically by effecting signal transduction and ion channels (256). Glucocorticoids activate cell membrane ion channels and decrease intracellular

calcium (257). They also activate phosphatidyl inositol 3-kinase leading to increased endothelial nitric oxide synthase activation (258). As discussed above glucocorticoids can bind to membrane receptors. See chapter 1 Figure 8 for an overview of glucocorticoid pathway.

The primary mechanism by which glucocorticoids work is by annexin-1 (lipocortin 1) synthesis. Annexin-1 suppresses phospholipase A2 blocking the production of eicosanoids (prostaglandins and leukotrienes) and inhibiting leukocyte recruitment. Moreover, glucocorticoids inhibit cyclooxygenase (COX1 and COX2). COXs are key mediators in the arachadonic acid pathway, activating prostaglandin synthesis.

Annexin-1 is a member of the annexin protein family that binds phosophlipids when Ca^{++} is present (259). In homeostasis, annexin-1 is present in PMNs, Mϕ, and monocytes (260). Following activation during leukocyte recruitment in acute inflammation, annexin-1 is released from the cells (261). After secretion, annexin-1 counter-regulates the activities of innate immunity (production of inflammatory mediators). Glucocorticoids increase the levels of annexin-1 expression by PMNs and monocytes (262, 263); the exact mechanism by which this is controlled is not understood. Glucocorticoids inhibit gene expression of pro-inflammatory factors, such as IL-1, IL2, TNFα, IFNγ, NOS, and adhesion molecules (264-267) important in the initiation and mediation of acute inflammation.

Glucocorticoids are potent anti-inflammatories in chronic inflammation. They can repress T cell activation. In contrast to the effects of glucocorticoids on annexin-1 in acute inflammatory cells (PMNs and monocytes), in chronic inflammation glucocorticoids inhibit annexin-1 on T cells (268). Annexin-1 increases expression in T_H cells after activation, increasing the production of AKT and extracellular signal-regulated kinases (ERK), thereby increasing T cell proliferation, differentiation and activity. Because glucocorticoids inhibit annexin-1 in this process, T cell proliferation is reduced as is their activity in ameliorating inflammation. As above,

glucocorticoids inhibit gene expression of pro-inflammatory factors; this plays a role in chronic inflammation via a T_H2-type response. Glucocorticoids have also been shown to be antagonists to COX (mainly COX2), by stabilizing COX2 mRNA and mediating prostaglandin synthesis (269) in chronic inflammation.

As discussed above, glucocorticoids are widely used as intra-articular injections in the treatment of symptoms in OA. The results are short lived (270), and there are adverse effects associated with them (immunosuppression and flares after injection causing cartilage damage). The majority of studies that have investigated the efficacy of glucocorticoids in OA have focused on pain management, making their anti-inflammatory potential less well known. Corticosteroids are often used to treat acute inflammation post-injury in order to allow the patient to return to sports. Because of patients' rapid return to sports, it is difficult to assess whether the steroids were an effective long term anti-inflammatory preventing later development of OA, or simply a short term analgesic and anti-inflammatory to allow a rapid return to sports and further damage and inflammation follows.

6.2. IL-1Ra/Anakinra

The use of biological molecules with targeted anti-inflammatory properties is an interesting and novel approach to control pro-inflammatory cytokine production and activity. IL-1Ra has elicited much attention for use in inflammatory disorders, including arthritis. IL-1Ra has had an effect in many models of various disorders, dysfunctions and injuries (for a summary see (197). Treatments with IL-1Ra have been efficacious in minimizing the effects of RA in both animal models and human subjects. *In vitro* studies of cartilage explants from RA-induced New Zealand White (NZW) rabbits treated with human recombinant IL-1Ra suppressed GAG release and synthesis, inhibited PGE2 production and inhibited collagenase production (271). Subsequent studies investigated human recombinant IL-1Ra treatment *in vivo*; intra-articular

injections in RA-induced NZW rabbits inhibited synovitis and articular cartilage loss (272, 273). IL-1Ra gene therapy in rat models of RA suppressed endogenous IL-1 and decreased joint swelling (274). When IL-1Ra was injected subcutaneously in patients with RA in a series of clinical studies there was a reduction in inflammatory cell infiltration and decreased radiological progression of the disease with little to no adverse events (275-279). IL-1Ra is being used clinically in RA patients who fail to respond to other older therapeutics.

While treatment with IL-1Ra (Anakinra) is promising, there are several significant differences between RA and OA which raises the question of whether this treatment would be effective in OA as well. Caron *et al* (280) found a chrondroprotective effect in experimentally induced OA in dogs after intra-articular injections of IL-1Ra. They further confirmed the suppression of OA with IL-1Ra using gene therapy (281) and showed a marked reduction in rabbit osteophyte growth (282). Zhang *et al* (283) found similar results using gene transfers in NZW rabbits, as did Frisbie *et al* (284). A clinical trial of safety and efficacy of Anakinra in OA patients found that while Anakinra (50mg or 150 mg) was well tolerated, there were no improved outcomes compared to placebo (285, 286). However, patients with an ACL tear receiving a single intra-articular injection of Anakinra (150mg) had markedly improved KOOS (knee injury and OA outcome score) and lower synovial fluid IL-1 levels compared with saline treated controls (287). While human studies are scarce, IL-1Ra drugs present an interesting avenue by which to prevent or slow the development of OA.

7. ANIMAL MODELS OF OSTEOARTHRITIS

There are a variety of animal models of OA being used; these include mechanical models, immobilization models and drug induced models in dogs, rats, guinea pigs, rabbits, sheep, cats, horses and mice. An ideal model of OA should be reproducible and result in disease in a reasonable time course. It should have distinct early, middle and late disease, should use an inexpensive mammal with easy handling and multiple forms of analysis, and structure and function should correlate.

7.1. Induction of OA

As described above there are several methods of inducing OA in animals. This can be done by injecting substances directly into the joint, by immobilizing the joint, or by surgically damaging the joint.

7.1.1. Intra-articular injections

A variety of substances can be injected into the joint in order to induce local joint inflammation and later cartilage damage suggestive of OA. Enzymes, such as papain, collagenase, trypsin, and hyaluronidase induce reproducible cartilage damage (288); however, physiologically proteolysis does not precede OA disease onset as it does in these models, and therefore they are likely inappropriate to study biological mechanisms of OA. IL-1 (289) and TGFβ injections (290) also induce local joint inflammation, again these fail to mimic physiological OA and therefore are not suitable for studying pathophysiology. Monosodium iodoacetate (MIA) injections cause cartilage damage via chondrocyte apoptosis and local inflammation; similar to the previously described modalities this fails to replicate the physiologic OA process. Lastly, quinolones can be used to induce OA by damaging articular cartilage; however, deep cartilage zones survive (291) unlike human OA. None of these reagents mimic human OA.

7.1.2. Immobilization

Joint immobility leads to cartilage atrophy (292) and OA like changes. The mechanism by which this occurs is via nutritional deprivation of the cartilage, which is not characteristic of OA, leading to chondrocyte necrosis.

7.1.3. Surgically-induced OA

All previous models of surgically induced OA work by destabilizing the knee and causing gross mechanical joint changes. There are a variety of surgeries done: ACL transection, meniscectomy, mixed injury, and focal cartilage defect (293, 294). ACL transection can be done in various animals and is reproducible, but has a greater severity of OA and quicker disease onset than humans who have an ACL tear (295-297). Meniscectomy, similarly, has higher disease severity and quicker onset than occurs in humans with the same injury. They do, however, produce reproducible disease, for example 100% of meniscectomized sheep develop OA within 3 months of injury (298-300). Combination/mixed injuries result in similar results as the previous two surgeries (154, 301, 302).

7.2. Animals

Each animal has its own advantages and disadvantages as a model of OA. Mice and rats are inexpensive, easy to use, and the whole joint can be visualized histologically. They are also well characterized and controlled genetically. While dogs, sheep and horses have larger joints which can be visualized with MRI and can have arthroscopic surgery, they are expensive and more difficult to work with. Mouse, rat and guinea pig models can be surgically or chemically induced (291, 303, 304). Dogs and sheep are acceptable for surgical destabilization models of OA (154, 296, 300-302, 305), but are inappropriate for intra-articular injection-induced OA.

A commonly used model that has been deemed as appropriate for studying secondary OA is a rabbit model. Rabbits have been used extensively as injury models for OA because of

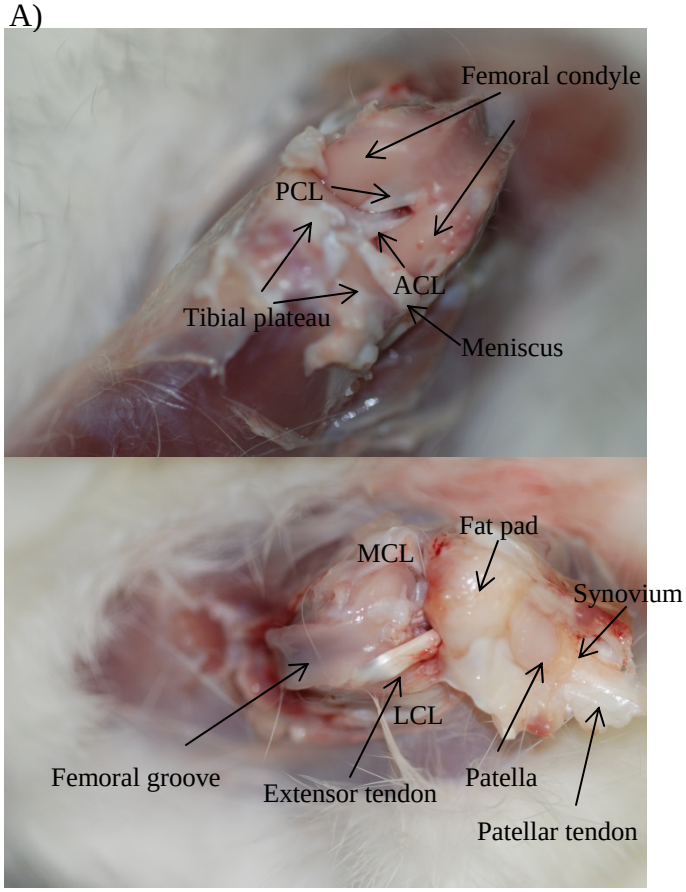
their ease of use, low cost and reproducibility. Rabbits are still considered a good model (295, 306), and possibly a more appropriate model for studying certain aspects of biochemistry and molecular biology. For this project they are an especially valuable model for exploring the role of inflammation, the early onset of inflammation and therapeutic interventions. The balance of cost, feasibility of experimentation, and the advantages for molecular work makes rabbits a good laboratory model for examining the molecular aspects of inflammation associated with secondary OA.

8. FIGURES

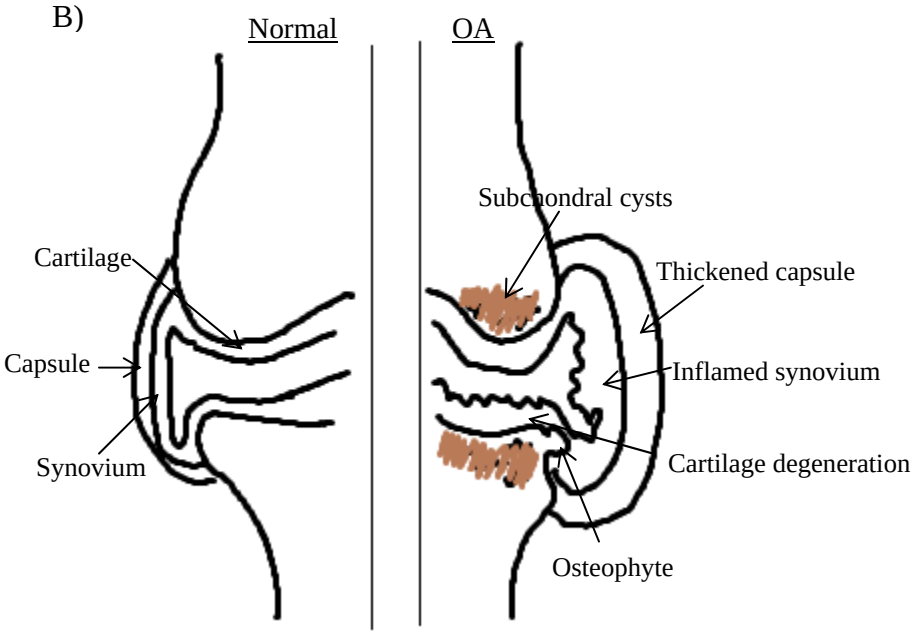
8.1. Figure 1 Overview of anatomy of the knee joint and OA

Figure 1: Schematic representations of A. The normal anatomy of the knee joint in a dissected rabbit knee, including the PCL, ACL, LCL, MCL, fat pad, patellar tendon, synovium, extensor tendon, meniscus and bony structures, and B. Gross OA of the knee, demonstrating: synovial inflammation, joint capsule thickening, cartilage damage, and osteophyte formation compared with a cartoon of the normal knee joint.

A)

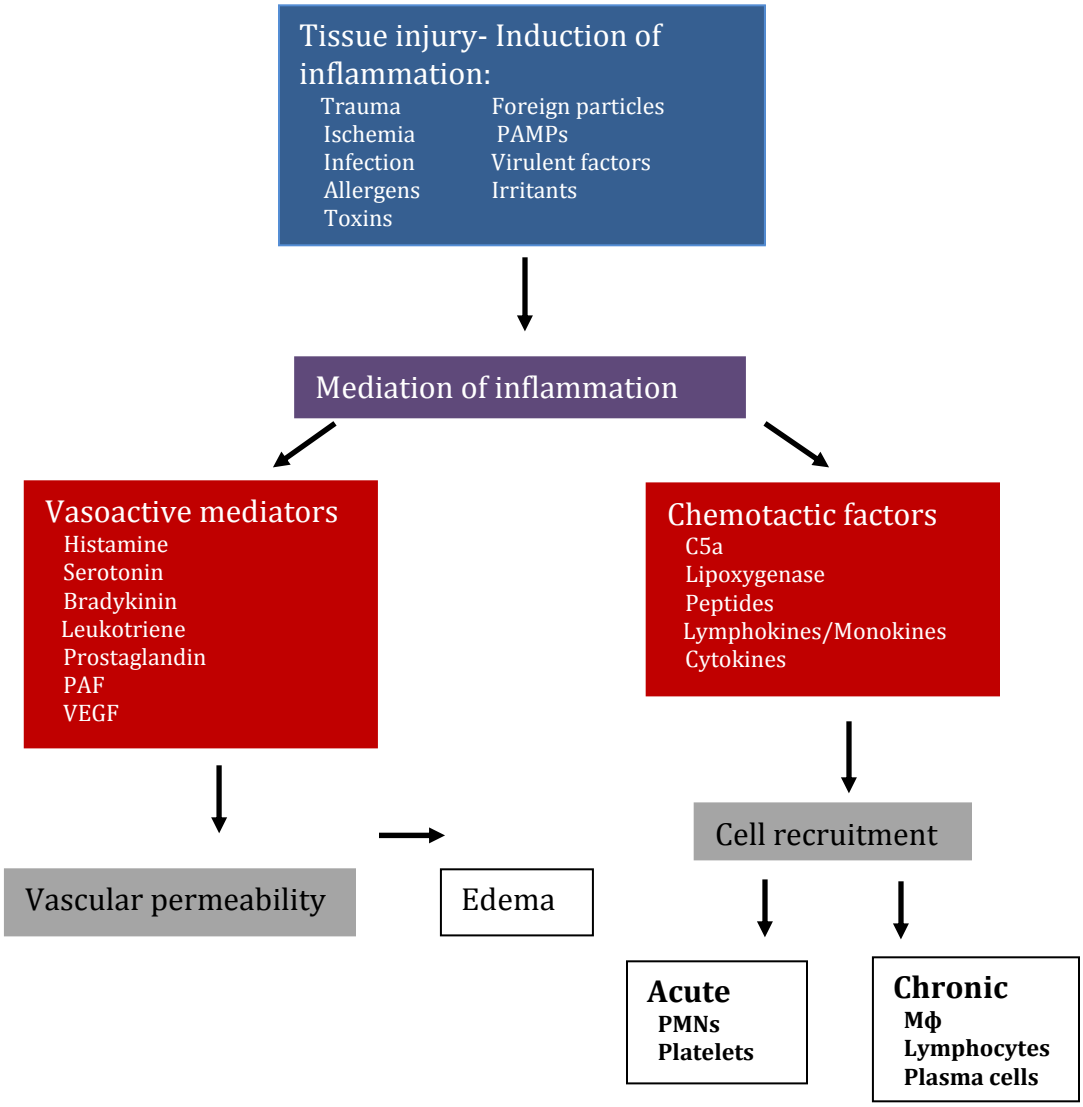

Femoral condyle
PCL
ACL
Tibial plateau
Meniscus
Fat pad
MCL
Synovium
LCL
Femoral groove
Extensor tendon
Patella
Patellar tendon

B)


Normal
OA
Cartilage
Capsule
Synovium
Subchondral cysts
Thickened capsule
Inflamed synovium
Cartilage degeneration
Osteophyte

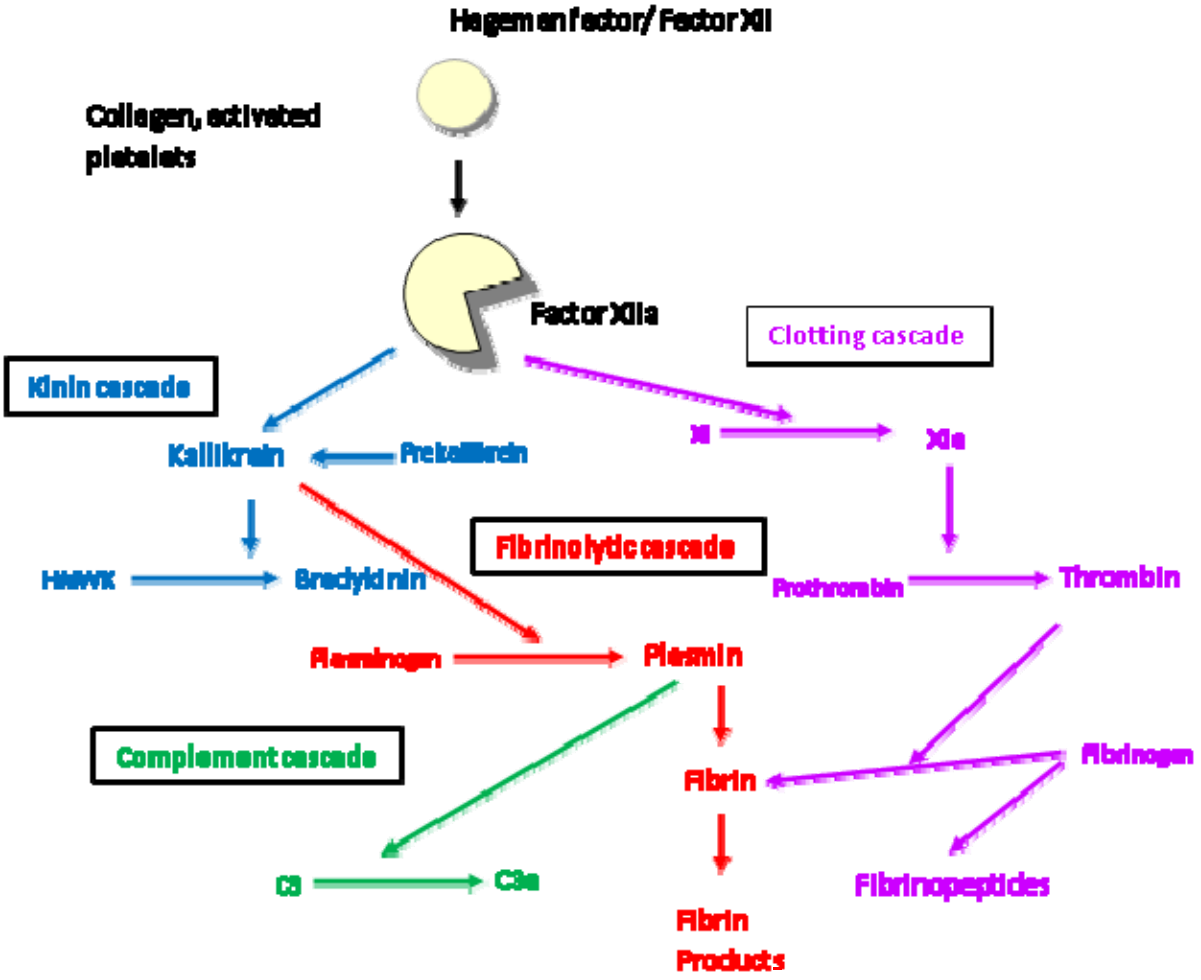
8.2. Figure 2 Inflammatory inducers and mediators

Figure 2. An overview of inflammatory inducers and mediators and their division into acute inflammation and chronic inflammation. After induction of inflammation vasoactive mediators and chemotactic factors increase vascular permeability and recruit cells to the site of injury. While PMNs and platelets are involved in acute inflammation, lymphocytes, macrophages and plasma cells are involved in chronic inflammation.


Tissue injury- Induction of inflammation:
Trauma
Ischemia
Infection
Allergens
Toxins
Foreign particles
PAMPs
Virulent factors
Irritants
Mediation of inflammation
Vasoactive mediators
Histamine
Serotonin
Bradykinin
Leukotriene
Prostaglandin
PAF
VEGF
Chemotactic factors
C5a
Lipoxygenase
Peptides
Lymphokines/Monokines
Cytokines
Vascular permeability
Edema
Cell recruitment
Acute
PMNs
Platelets
Chronic
Mϕ
Lymphocytes
Plasma cells

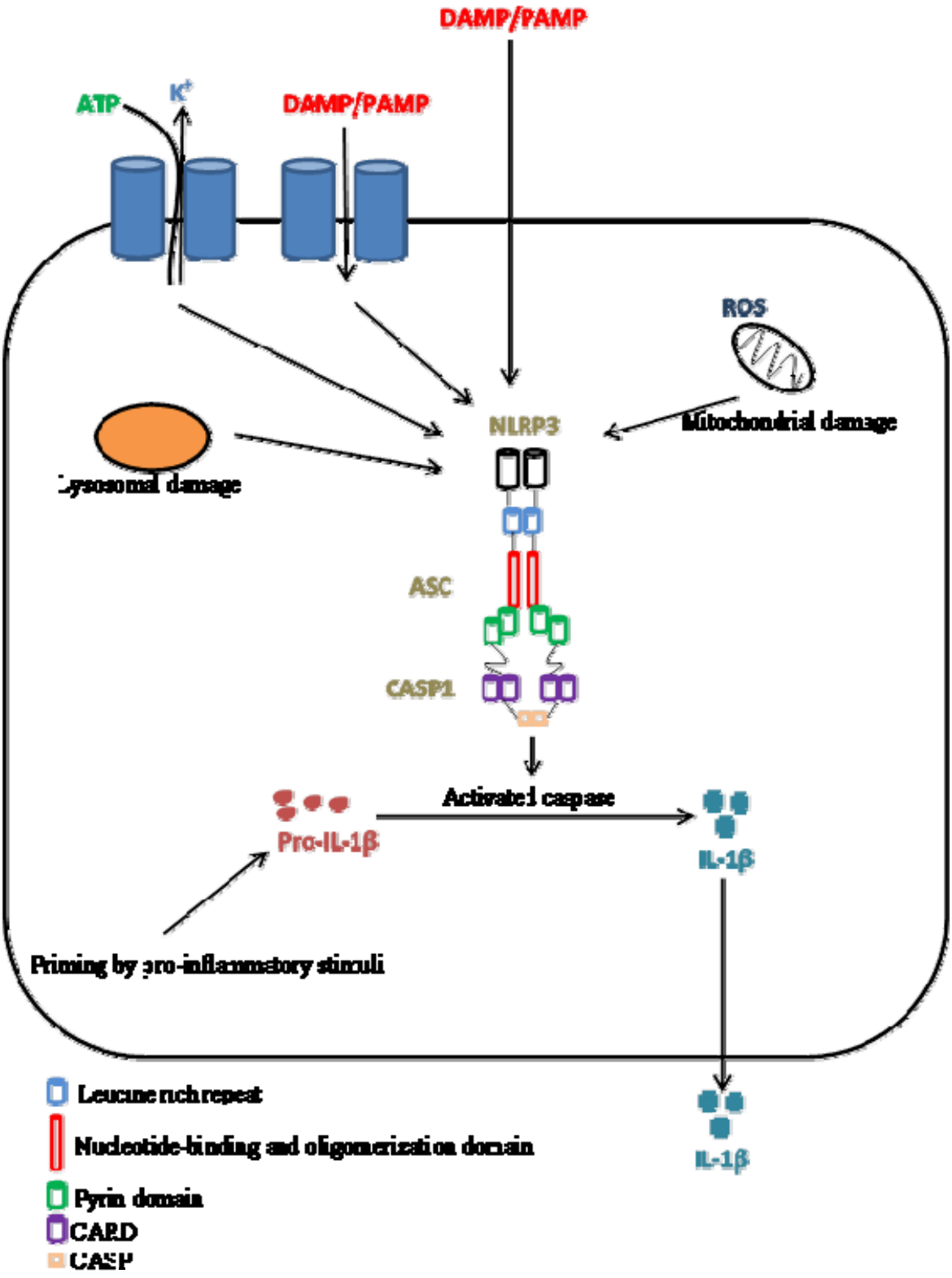
8.3. Figure 3 Endothelium damage cascade

Figure 3. Hageman factor activation after ECM contact signals four plasma regulator systems that result in endothelial damage. (Four cascades: kinin cascade, complement cascade, fibrinolytic cascade, and clotting cascade).


Hagemen factor/ Factor XII
Collagen, activated platelets
Factor XIIa
Clotting cascade
Kinin cascade
Kallikrein
Prekallikrein
XI
XIa
Fibrinolytic cascade
HMWK
Bradykinin
Prothrombin
Thrombin
Plasminogen
Plasmin
Complement cascade
Fibrin
Fibrinogen
C3
C3a
Fibrinopeptides
Fibrin Products

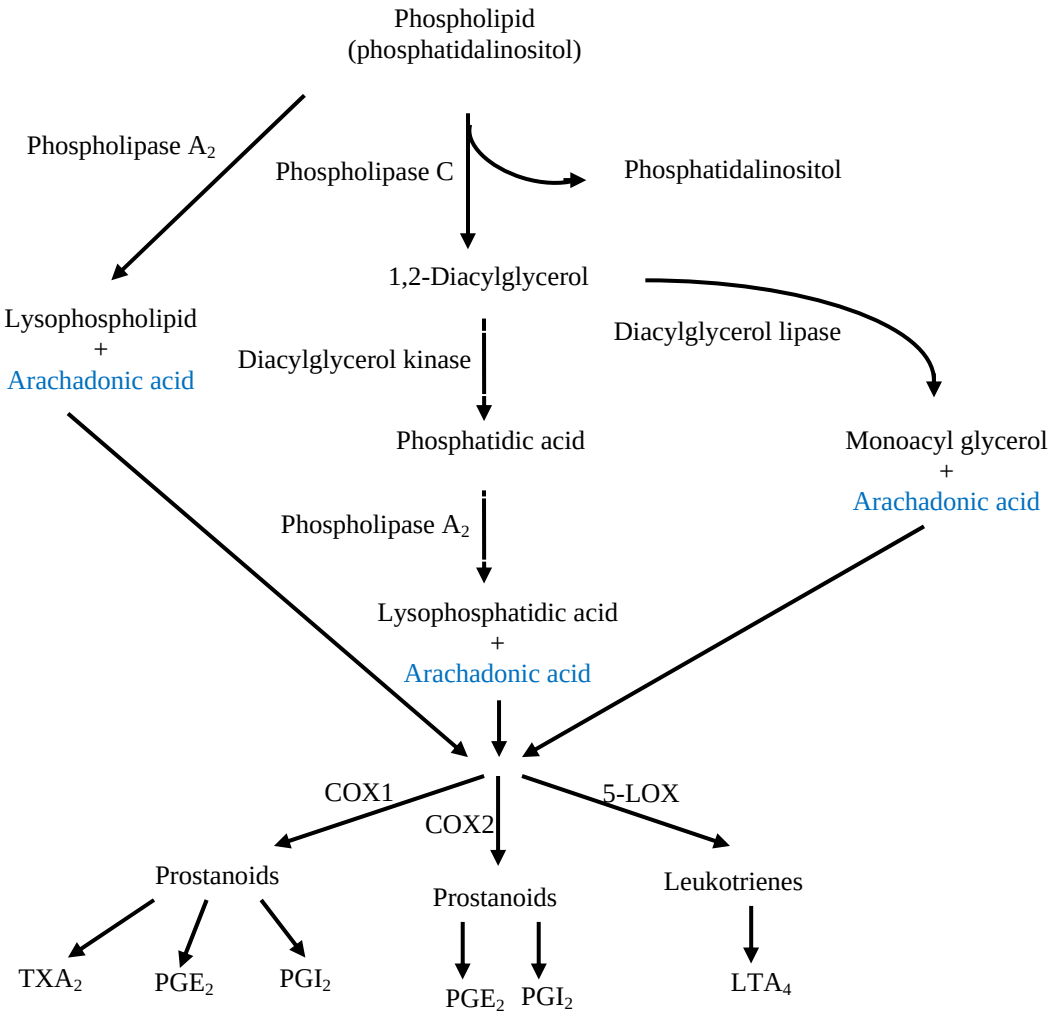
8.4. Figure 4 The inflammasome

Figure 4. Basic mechanism of activation of the NLRP3 inflammasome, via potassium efflux, ROS, and lysosome destabilization. ATP allows extracellular stimuli to enter the cell to activate NLRP3. Caspase-1 clustering causes autoactivation and caspase-1 maturation and secretion of IL-1β and IL-18.


DAMP/PAMP
ATP
K+
DAMP/PAMP
ROS
NLRP3
Mitochondrial damage
Lysosomal damage
ASC
CASP1
Activated caspase
Pro-IL-1β
IL-1β
Priming by pro-inflammatory stimuli
Leucine rich repeat
Nucleotide-binding and oligomerization domain
Pyrin domain
CARD
CASP
IL-1β

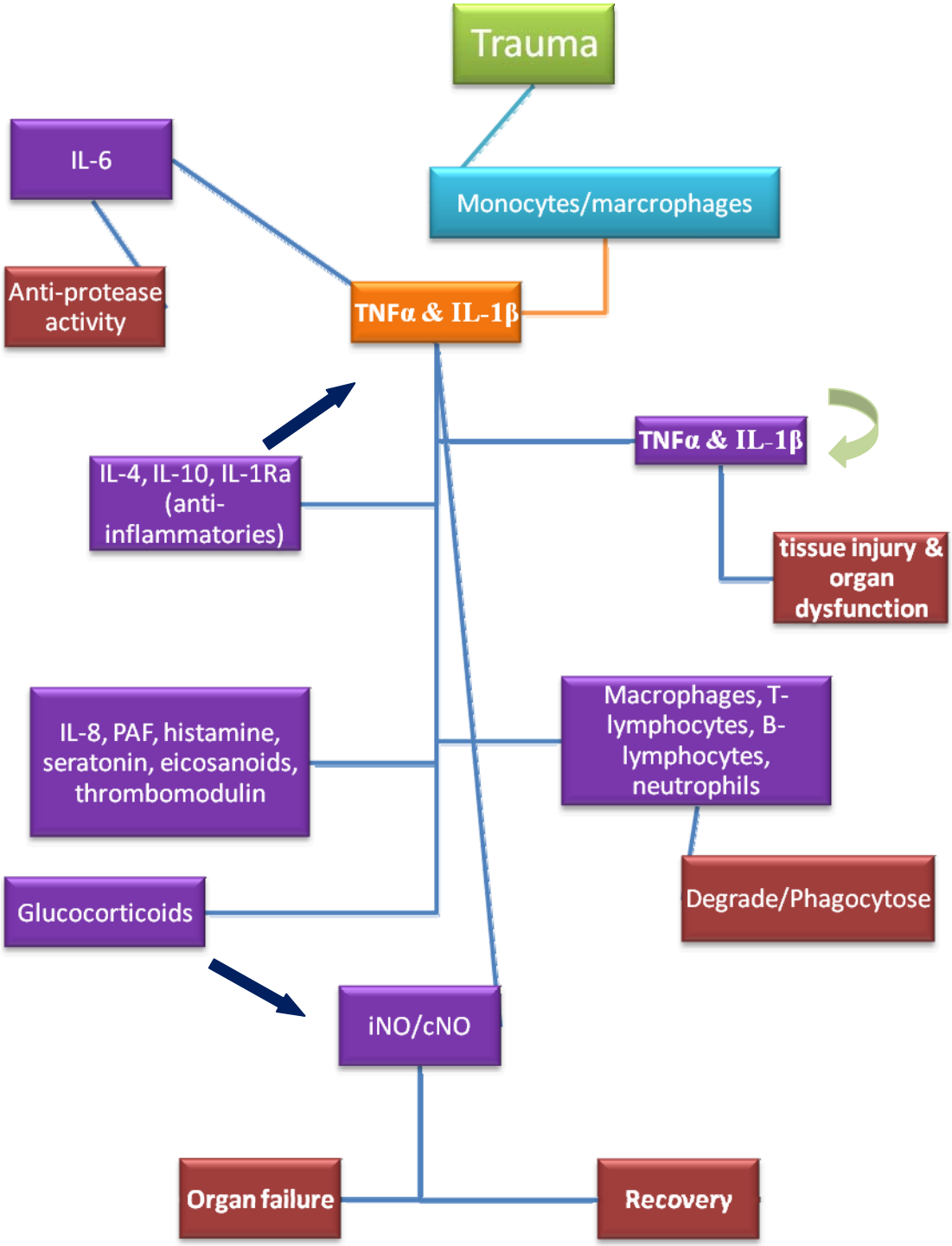
8.5. Figure 5 Arachadonic acid pathway

Figure 5. Release and fate of Arachadonic acid from membrane lipids (PLA_2). Arachadonic acid produces pro-inflammatory prostaglandins (PGE_2 and PGI_2), thromboxin (TXA_2), and leukotrienes or anti-inflammatory lipoxins. These are inducers of the acute inflammatory phase and act in a variety of tissue types.


Phospholipid
(phosphatidalinositol)
Phospholipase A_2
Phospholipase C
Phosphatidalinositol
1,2-Diacylglycerol
Lysophospholipid
+
Arachadonic acid
Diacylglycerol kinase
Diacylglycerol lipase
Phosphatidic acid
Monoacyl glycerol
+
Arachadonic acid
Phospholipase A_2
Lysophosphatidic acid
+
Arachadonic acid
COX1
COX2
5-LOX
Prostanoids
Prostanoids
Leukotrienes
TXA_2
PGE_2
PGI_2
PGE_2
PGI_2
LTA_4

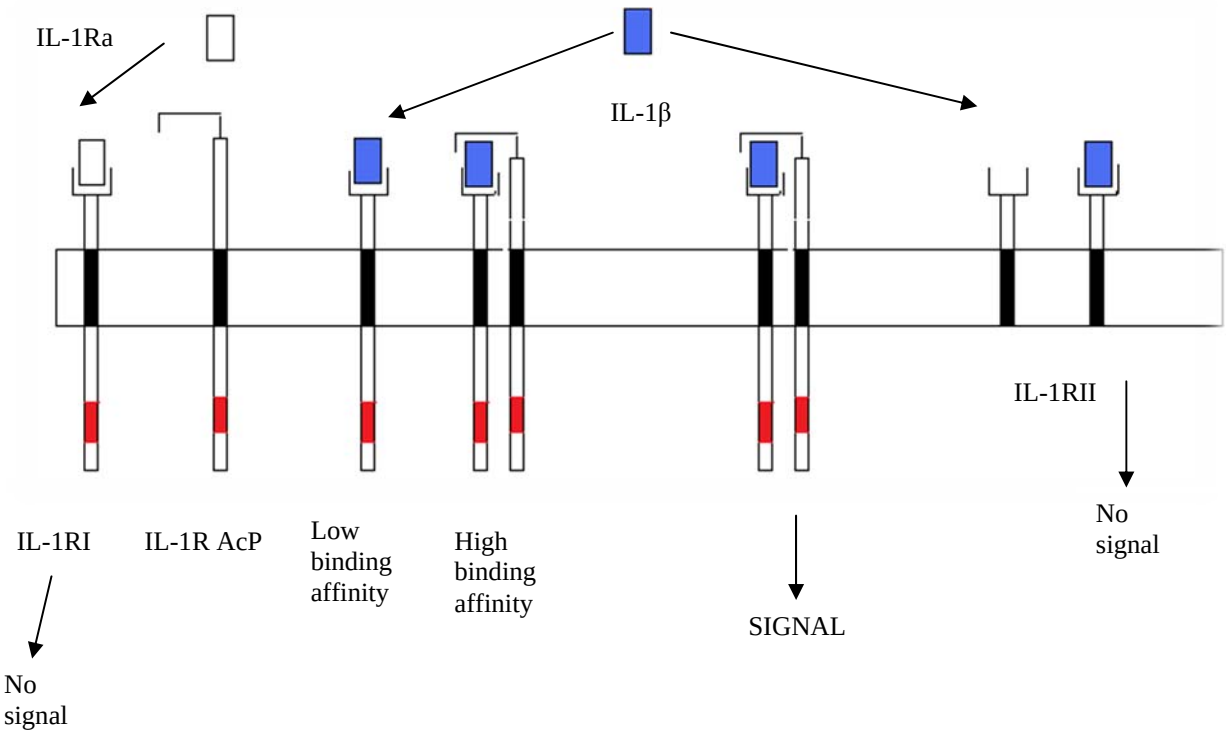
8.6. Figure 6 Inflammatory signal cascade

Figure 6: Inflammatory signal cascade post-injury. Trauma leads to leukocyte infiltration releasing pro-inflammatory cytokines into the site and propagating a variety or both pro- and anti-inflammatory processes. Connecting lines represent activation, dark blue arrows represent inhibition, and the light green arrow represents auto/paracrine loop.


Trauma
IL-6
Monocytes/marcrophages
Anti-protease activity
TNFα & IL-1β
TNFα & IL-1β
IL-4, IL-10, IL-1Ra (anti-inflammatories)
tissue injury & organ dysfunction
IL-8, PAF, histamine, seratonin, eicosanoids, thrombomodulin
Macrophages, T-lymphocytes, B-lymphocytes, neutrophils
Degrade/Phagocytose
Glucocorticoids
iNO/cNO
Organ failure
Recovery

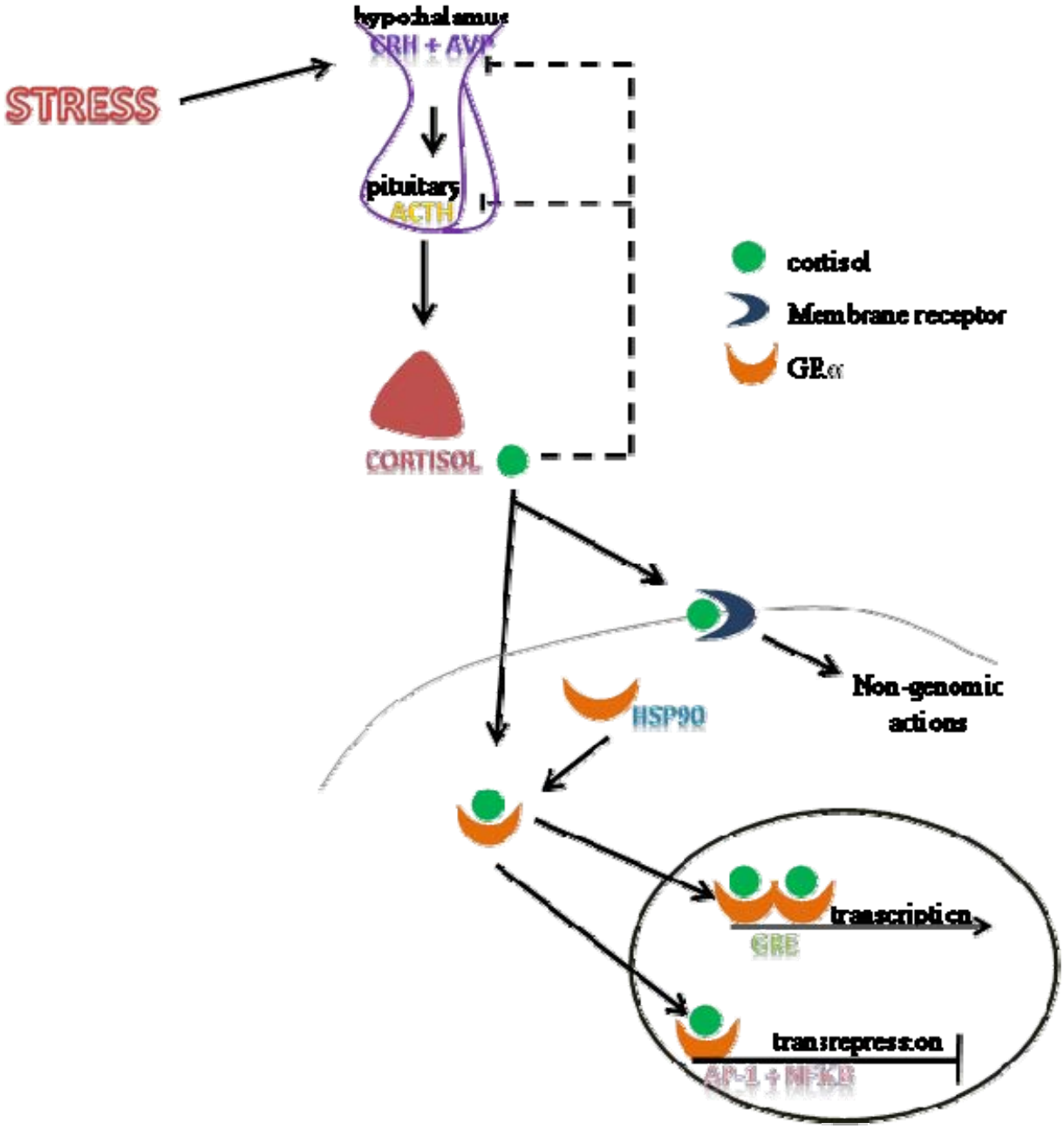
8.7. Figure 7 IL-1β and IL-1Ra signalling

Figure 7: IL-1β and IL-1Ra binding and signalling properties. IL-1 can bind to both IL-1RI and IL-1RII receptors; binding to IL-1RI will induce a response, while IL-1RII acts as a decoy. IL-1Ra can only bind to IL-1RI, with no signal.


IL-1Ra
IL-1β
IL-1RII
No
signal
IL-1RI
IL-1R AcP
Low
binding
affinity
High
binding
affinity
SIGNAL
No
signal

8.8. Figure 8 Schematic of hypothalamic-pituitary-adrenal axis and glucocorticoid actions

Figure 8. Physiologic stress stimulates the hypothalamus to release corticotropic releasing hormone (CRH) and arginine-vasopressin (AVP) causing the release of ACTH from the anterior pituitary which then leads to cortisol to be released from the adrenals. Cortisol binds to either membrane receptors or intracellular receptors. Once bound to intracellular receptors genes are either transcribed or transrepressed. Cortisol feedbacks inhibiting further CRH/AVP and ACTH release.


STRESS
hypothalamus
CRH + AVP
pituitary
ACTH
CORTISOL
cortisol
Membrane receptor
GRα
Non-genomic actions
HSP90
transcription
GRE
transrepression
AP-1 + NF-KB

9. RATIONALE AND HYPOTHESES

Based on the evidence presented above I sought to develop a new surgical model of post-traumatic OA in a rabbit that did not destabilize the knee joint in order to isolate biological joint changes without gross mechanical instability as a confounder. After developing the model and pilot testing its ability to produce PTOA, I then aimed to investigate the mechanistic role of post-injury joint inflammation in creating cartilage changes consistent with PTOA by inhibiting various inflammatory mediators to both understand the pathophysiology of inflammation in PTOA and to begin to investigate possible therapeutic targets in this model.

The hypotheses tested were:

1) Based on the hypothesis that early post-traumatic inflammation that was induced by an intra-articular bone injury would cause early PTOA in rabbits, I speculated that drilling intra-articular holes into the femoral notch, without damaging the cartilage or ligaments, would lead to predictable early development of PTOA as compared with sham and normal controls.
2) Based on the hypothesis that PTOA in this rabbit model was caused by post-injury inflammation and could be prevented by blocking that inflammation, I sought to inhibit synovial inflammation in the same rabbit model by intra-articular corticosteroid injections (0.5mg/kg pre-op and every third day for three weeks). I hypothesized that this will inhibit joint inflammation and therefore protect joint cartilage. Also will demonstrate that this new model is an “inflammatory” model of OA**.**
3) I further hypothesized that the imbalance of IL-1β to IL-1Ra is the main mechanism for the development of OA and that IL-1Ra injections would thus prevent the development of PTOA in this model. This was based on IL-Ra

intra-articular injections (0.5mg/kg/day once daily) targeting IL-1β by competitively binding IL-1 receptors and preventing the ensuing inflammatory cascade. IL-1Ra injections will prevent the development of post-traumatic OA.

4) Finally, I hypothesized that leukocyte recruitment to the joint is a very early driver of inflammation and leukocyte inhibition by an intravenous injection of 500μl/kg tripeptide feG 5 minutes pre-operatively will inhibit leukocyte recruitment to the knee joint post-injury thereby prevent synovial inflammation and the development of PTOA.

See appendix 1 for an unrelated study examining the ultrastructure and molecular composition of the AM and PL bands of the ACL with maturation. The aim was to determine the differences in between AM and PL bundles of the ovine ACL during development.

Hypothesis:

I hypothesized that due to distinct functional roles of the AM and PL bundles the ACL is two discrete structures with different architecture and composition in both immature and mature sheep.

Chapter 2. NEW SURGICAL MODEL OF PTOA

1. ABSTRACT

Osteoarthritis (OA) is a leading cause of disability worldwide. I hypothesized that inflammation following isolated intra-articular bone injury can stimulate post-traumatic OA and developed a rabbit model to test that concept. Sixty female New Zealand White Rabbits were used. 26 experimental animals had two holes drilled into their right femoral-notch, 18 rabbits had sham surgery, and 16 were un-operated controls. Rabbits were euthanized in subgroups at 72hours, 3, 6, 9, and 52weeks. Knees were assessed grossly and tissues collected. Cartilage and synovium were analyzed with histology and qPCR and subgroups compared statistically. All surgical joints showed gross and histological (modified Mankin score) cartilage damage after surgery, with experimentals worsening with time ($p<0.05$). Cartilage qPCR showed 5-fold increases in TGFβ ($p <0.05$) expression at 72hours and 3weeks with 6-fold increases in MMP13 ($p <0.025$) expression at 72hours. By 6weeks, expression of these markers was similar to baseline levels. Synovial membrane thickening with increased cellularity was seen at both 9 and 52weeks ($p <0.05$). Short-term synovial inflammatory marker (IL-1β, ILRa, IL-6 and IL-8) expression was 3-4-fold increase in experimentals at 72hours ($p <0.01$) returning to baseline levels by 3weeks. Intra-articular bone injury creates early joint inflammation with some chronic synovial changes and progressive cartilage damage consistent with OA in adult rabbits. This model provides an exciting new avenue to potentially explore some relevant inflammatory drivers of OA without major mechanical variables.

2. INTRODUCTION

Osteoarthritis (OA) is a debilitating disease affecting 27 million Americans (307) and over 3 million Canadians (308). According to the World Health Organization (309) OA is the fourth leading cause of years lost to disability worldwide. With increasing numbers of the population suffering from this disease, it is key that we understand its processes to develop better treatments and prevention strategies.

Anterior Cruciate Ligament (ACL) injuries are a common cause of secondary knee OA. Unfortunately ACL reconstructions seem to do little to change the natural history of OA; over 50% of patients develop OA in their reconstructed knee (160, 161). The reasons for this remain obscure. It is thought that residual altered biomechanics, post-injury biological changes, or a combination of the two is causing OA to occur. Inflammation in the joint may be a driver or a combined stimulus with altered biomechanics for the development of OA (310).

Current animal models of secondary OA of the knee joint primarily involve either chemically induced or surgically induced OA. The latter destabilize the joint, altering mechanics and thus presumably damaging joint surfaces by abnormal loads on their surfaces (301, 311, 312). In models that cause mechanical abnormalities along with biological changes, separating the potential damaging effects of the biological changes alone have not been attempted. I created a surgical model of OA in which I could investigate the isolated effects of injury-induced inflammation without destabilizing the knee. My aim was to use a rabbit model (313, 314) and create an injury that would neither alter loading of cartilage, nor alter the mechanics of the joint. I tested the hypothesis that creating a bone injury by drilling into the non-load bearing inter-condylar notch would lead to secondary cartilage degradation, and thus to PTOA.

3. MATERIALS AND METHODS

3.1. Surgical methods and animal groups

To study cartilage and joint changes after inter-condylar drilling, sixty 1 year old female New Zealand White rabbits were used. Twenty six animals received experimental bone-injury surgery (72 hours (n=6), 3 weeks (n=6), 6 weeks (n=6), 9 weeks (n=6), and 52 weeks (n=2) groups) called BI animals; eighteen animals received sham arthrotomy surgery alone (3 weeks (n=6), 6 weeks (n=6) and 9 weeks (n=6) groups) called Shams; and sixteen animals served as non-operated normal controls (called Ctls) to account for inter-animal variability.

3.1.1. Inter-condylar drill hole surgery

Twenty six experimental rabbits underwent surgery to induce highly controlled bone injury; approved by the U of C Animal Care Committee. Animals were anaesthetized with Acepromazine Maleate (0.9mg/kg IV) as a pre-anaesthetic and isofluorane (2-2.5% inhaled) as surgical anaesthetic. Buprenorphine (0.01mg/kg SC) was given as an analgesic both pre- and post-operatively, and penicillin (180,000IU/kg SC) was given pre-operatively. A lateral arthrotomy between the extensor tendon and patellar tendon was performed to gain access to the femoral notch. Deflecting the fat pad and avoiding both cruciate ligaments, using a drill guide, two 1.1mm drill holes (ECT® internal fracture fixation drill, Zimmer, Warsaw, IN) were drilled 7mm apart posterior to the chondral margins, to a depth of 10mm in order to create consistent injuries in non-load bearing locations. The hole angle and depth ensured penetration of the marrow cavity of the femur. The fat pad was then reduced and the incisions closed in layers. Animals were allowed immediate cage activity after surgery until they were sacrificed in groups of 6 rabbits at 72 hours (72h), 3 (3w), 6 (6w), or 9 weeks (9w). To examine the longer term effects of this model descriptively, two additional rabbits were sacrifice at 52 weeks (52w). See appendix 2 for pictures of the surgical approach.

3.1.2. Sham surgery controls (Sham)

Based on the histological data at 3w, 6w, and 9w that showed minimal cartilage changes, Shams were not considered necessary at the 72h time interval. Eighteen Sham rabbits (n=6/ group) were similarly anesthetised and a similar surgical approach to their joint was used to gain access to the femoral notch. The femoral notch was lightly touched with forceps alone. As in the experimental group, the fat pad was reduced and incisions were closed. Sham groups were sacrificed at 3, 6, and 9 weeks post-surgery. (Note: since there were only minimal gross changes seen in the 3, 6 and 9 week sham groups there were no 72 hour or 52 week sham animals).

Sixteen non-surgical normal control (Ctl) animals were analyzed concurrently. At the time of euthanasia all joint tissues were isolated and collected from each compartment of both right and left knees. The joint gross morphology was assessed qualitatively for osteophytes, cartilage lesions, cartilage discolouration and cartilage fibrillation by two experienced observers. Synovium was sampled from the suprapatellar area and the femoral groove. Cartilage samples were taken from the centre of six different areas: patella, femoral groove, lateral and medial femoral condyle, and lateral and medial tibial plateau. Samples were flash frozen in isopentane on dry ice or whole in liquid nitrogen.

3.2. Histology

To determine the impact of the drill hole surgery, cartilage was embedded in Tissue-Tek® O.C.T.™ (Sakura Finetek USA, Inc., Torrance, CA) and cryosectioned (5μm) in order to do both chemical stains and immunohistochemistry on the samples. Slides were stained with Safranin-O/Fast-green, and graded blind by one observer on two occasions with a modified Mankin score (315) (Appendix 3 Table 1). I chose a Mankin score as opposed to the OARSI score as the OARSI score was for ACL defiencies and as previous studies in the lab were done with Mankin they would not be comparable. See Appendix 4 for an example of histological

grades. A minimum of six sections from each area of each joint were analyzed and scores were summed across the areas.

The synovium was cryosectioned (10μm) and stained with Hematoxylin and Eosin, and slides were graded blind by one observer on two occasions for cell infiltrate with a scheme eliminating non-infiltrate evaluation (316). Synovial membrane thickness was analyzed using Image J software (NIH, USA) taking an average thickness of three sections.

3.3. Real-time reverse transcriptase polymerase chain reaction (q-PCR)

The cartilage from the tibial plateaus (pooled) and femoral condyles (pooled) of Ctl, and 72h, 3w, 6w and 9w experimental animals were examined for expression of transforming growth factor β (TGFβ), critical in cartilage maintenance, and matrix metalloproteinase-13 (MMP13), a key player in cartilage degeneration, using qPCR as described by Reno *et. al.*(317). Samples were run in duplicate. RNA was extracted using a Qiagen RNeasy kit (Qiagen Sciences, Germantown, MD), treated and quantified with DNAse-I. 1mg of total RNA was transcribed to a single stranded CDNA with Qiagen Omniscript RT kit (Qiagen Sciences, Germantown, MD). Primers were made and validated for TGFβ and MMP13 (Appendix 3 Table 2). iCycler Thermal Cycler (BIO-RAD, Hercules, CA) was used for amplification and detection after SYBR green supermix (BIO-RAD, Hercules, CA), molecular water, and forward and reverse primers were mixed with the samples. Gene expression was normalized to 18S mRNA and iCycler IQ Optical System Software version 3.0a (BIO-RAD, Hercules, CA) was used to quantify the results.

The synovium was also examined for changes in mRNA expression of 5 inflammatory factors: IL-1β, IL-1Ra, IL-6, IL-8 (interleukins) and tumor necrosis factor α (TNFα) (Appendix 3 Table 2) using the method above.

3.4. Micro Computer Tomography (μCT)

To confirm the gross observation that the drill holes became 'sealed', the bone microarchitecture was assessed with µCT (318). The right distal femurs (Ctl and experimental animals) were µCT scanned *ex-vivo* (µCT 35 Scanco Medical AG, Brüttisellen, Switzerland). A volume of interest of 400 slices was acquired from the distal end of the femur extending proximally up the shaft with a nominal isotropic resolution of 30 microns covering a range of 12 mm scan length from the distal end. The X-ray source had an energy level of 55 kVp, a current of 145 mA, and an integration time of 100 ms. User-defined contours were drawn on the images to define the cortical and trabecular regions (319). The images were Gaussian filtered (sigma of 0.8, support of 1.0) to reduce noise and the trabecular compartment was isolated from the image by subtracting the cortical region (parameters previous published (320, 321).

3.5. Statistics

Statistical analysis of cartilage Mankin scores was performed with Kruskal-Wallis and Friedman with Tukey's HSD, and ANCOVA with Bonferroni post-hoc (CI=95%). Scores were also compared between joint locations and subscores with ANCOVA with Bonferroni post-hoc (CI-95%). Synovial section grades were analyzed with a UNIANOVA with LSD post-hoc (CI=95%), and membrane thickness with a 2 tailed t-test. All qPCR data were analyzed using an MANOVA with Bonferroni post-hoc and confirmed with a Kruskal-Wallis test with Tukey's HSD post-hoc. All tests used unequal variance assumptions and non-parametric statistics were done to confirm results in order to account for group size differences and variability. A $p < 0.05$ was considered significant (SPSS; IBM, Armonk, NY).

4. RESULTS

During the course of the study animals appeared healthy, they were active in their cages and they ate and drank normally. Surgical animals regained baseline Ctl activity moving around cage within 6 hours after surgery (not quantified).

4.1. Gross and histological data

Synovial fluid was bloody at 72h, but there were minimal signs of cartilage damage. Signs of gross surface damage were apparent by 3w and cartilage showed some fibrillation and discolouration in all BI (experimental) animals, but minimal damage in Shams. By 6w post-surgery cartilage surfaces were roughened and osteochondral growths were beginning to form with consistent cartilage discolouration, and focal defects in all experimental rabbits. By 9w, gross cartilage damage was apparent on both the tibia and femur and there were large osteochondral growths and focal cartilage defects on the femur in all experimental animals. By 52w, articular cartilage was more severely worn and roughened, and large osteochondral growths had formed on both the femur and the tibia (n=2). Contralateral limbs and Sham animals at 3w, 6w and 9w showed minimal gross changes.

These gross findings of cartilage damage were supported by blinded histology. At 72h histological analysis of the knee cartilage from BI (experimentals) was slightly abnormal. At 3w, 6w and 9w histology of experimental joints showed chondrocyte cloning, surface damage and a loss of safranin-O staining. By 52w there was little to no safranin-O staining, and the cartilage was more severely damaged. There were no differences between subscores nor joint locations despite gross appearance differences of the femur ($p>0.05$), therefore these scores were pooled and averaged for a total joint score for each animal. At 72h, Mankin scores of the right limb were significantly higher than normal Ctl animals ($p = 0.02$) and at 72h contralateral left limb ($p = 0.03$), and significantly lower than other experimental animals ($p <0.01$) (Fig. 1). 3w, 6w and 9w

experimental Mankin scores were also significantly higher than normal Ctl animals ($p < 0.05$) and their respective contralateral limbs ($p < 0.005$) (Fig. 1). At 52w, the two right limb surgical joint cartilage scores were significantly higher than the scores of all the other animals ($p < 0.001$), as well as higher than their left contralateral limbs ($p < 0.001$) (Fig. 1). Sham animal scores were significantly different from 3w, 6w, 9w and 52w experimental animals ($p < 0.025$); however, they were not significantly different than the 72h experimental animals, but were higher than the normal Ctl animals (Fig. 1a).

By gross and histological examination, the synovial membrane was significantly ($p < 0.005$) thickened in experimental animals (0.54 ± 0.2) at 9w and 52w (Fig 2a-d). There were also significantly more cells at 9w ($p = 0.03$) and 52w ($p = 0.01$) after surgery in the synovium seen by graded histological sections (Fig 2e).

4.2. Molecular Effects

mRNA expression of MMP13 of the tibial plateau (pooled medial and lateral) was significantly higher in the surgical limb of experimentals 72h ($p < 0.025$; Fig. 3a); expression was not different from baseline values by 3w. mRNA expression of TGFβ of the tibial plateau (pooled medial and lateral) was also significantly higher in the surgical limb at 72h and 3w compared with the contralateral limb, normal Ctl animals, and other experimental groups ($p < 0.05$; Fig. 3b), and at baseline levels by 6w. While the tibial plateau showed significant differences in expression, there were no differences in TGFβ or MMP13 expression of the femoral condyles (pooled medial and lateral).

In the synovium, mRNA expression of IL-1β, IL-1Ra, IL-6, and IL-8 was significantly increased at 72h ($p < 0.01$; Fig. 4a-d); however, TNFα levels were unchanged (Fig. 4e). There were no significant differences in levels of synovial expression at later time points.

4.3. Healing of Drill Holes

Macroscopically, drill holes in experimental animals showed no visible healing at 72h but by 3w they had all filled with early callus. At 6w and 9w holes were completely filled with a firmer fibrocartilagenous callous. By 52w the holes were no longer apparent (Figure 5). By 52w there was no difference from normal Ctl joints, confirming that the holes had healed.

5. FIGURES

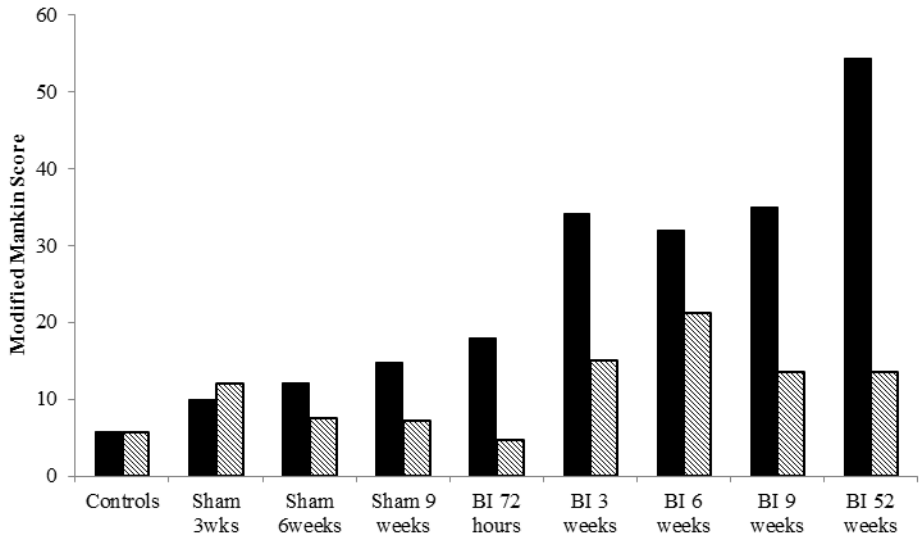
5.1. Figure 1 Cartilage histology

Figure 1. a) Cartilage histology Mankin scores of surgical limbs (black box), and contralateral limbs (hashed boxes). Mankin scores were worse with surgery and worse over time. Sham animal scores were part way between non-surgical controls (Ctl) and experimentals (BI).

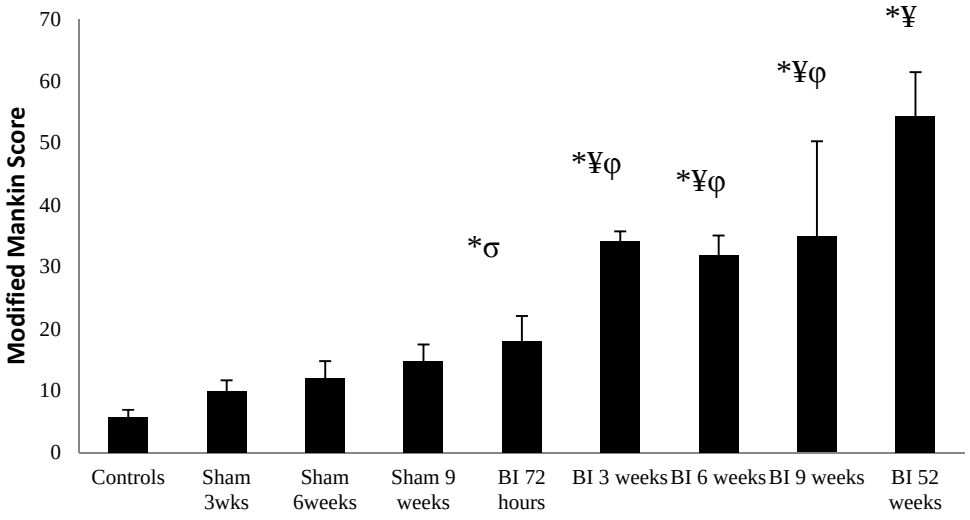
b) Cartilage histology Mankin scores of right surgical limb. *= significant difference from controls; ¥= significant difference from Shams (3, 6 and 9 weeks); φ= significant difference from 52 weeks; σ= significant difference from experimental (BI; 3, 6, 9 and 52 weeks) of $p<0.05$.

c) Cartilage histology scores of all experimental animals; (surgical limbs = black boxes) (contralateral limbs = hashed boxes). §= significantly different from contralateral limbs $p< 0.05$.

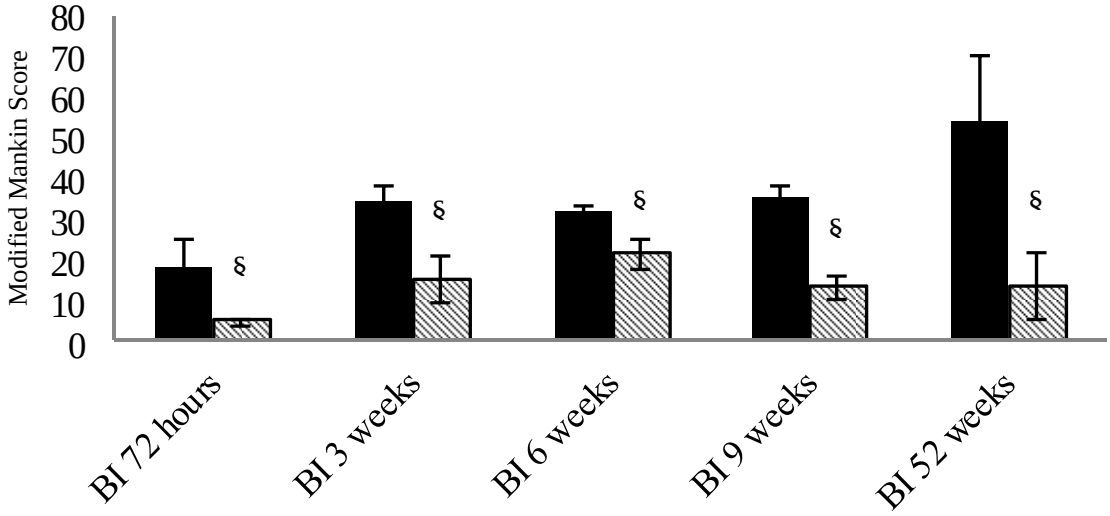
a)


Modified Mankin Score
60
50
40
30
20
10
0
Controls
Sham 3wks
Sham 6weeks
Sham 9 weeks
BI 72 hours
BI 3 weeks
BI 6 weeks
BI 9 weeks
BI 52 weeks

b)


Modified Mankin Score
70
60
50
40
30
20
10
0
*σ
*¥φ
*¥φ
*¥φ
*¥
Controls
Sham 3wks
Sham 6weeks
Sham 9 weeks
BI 72 hours
BI 3 weeks
BI 6 weeks
BI 9 weeks
BI 52 weeks

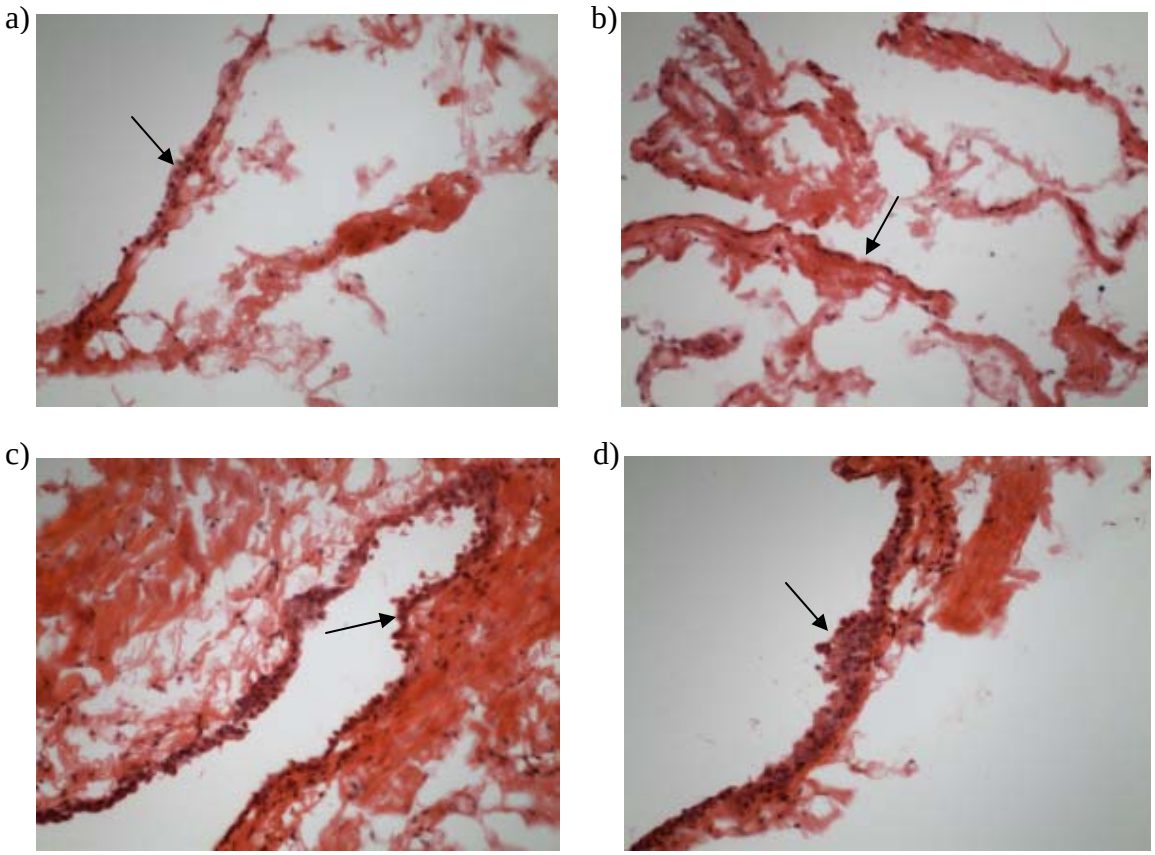
c)


Modified Mankin Score
80
70
60
50
40
30
20
10
0
§
§
§
§
§
BI 72 hours
BI 3 weeks
BI 6 weeks
BI 9 weeks
BI 52 weeks

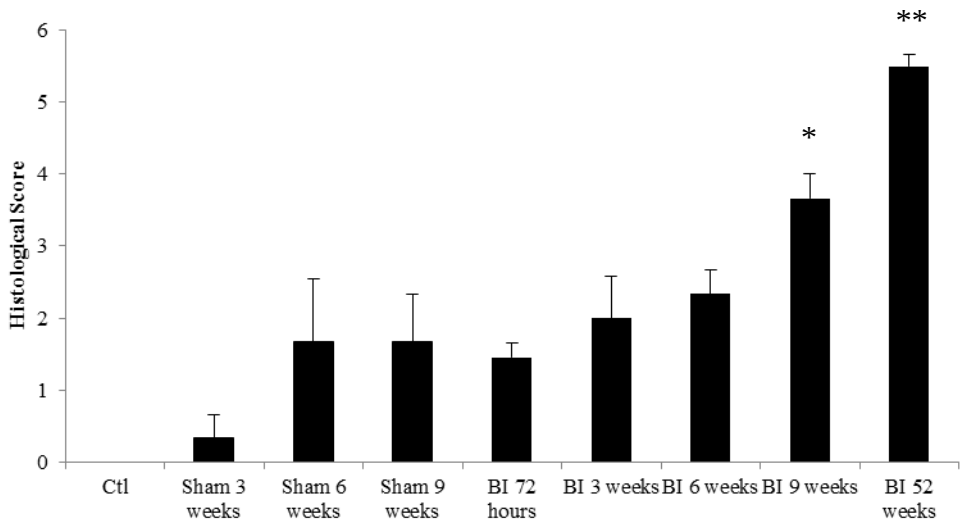
5.2. Figure 2 Synovium histology

Figure 2. a-d) Representative sections (20x magnification) of synovium demonstrating synovial membrane thickening over time. Experimental animals had significantly ($p < 0.005$) thickened membranes (0.54 ± 0.2) compared to normal controls (0.15 ± 0.04). a= experimental 72 hours; b = experimental 3 weeks; c = experimental 9 weeks; d = experimental 52 weeks. Note controls and shams were unremarkable for any membrane thickening (arrows are indicating synovial membrane).

e) Graph representing cell numbers scored with a modified synovial scoring system (316) in histological sections of synovium. * represents significance of $p<0.05$, and ** represents significance of $p<0.01$.


a)
b)
c)
d)

e)

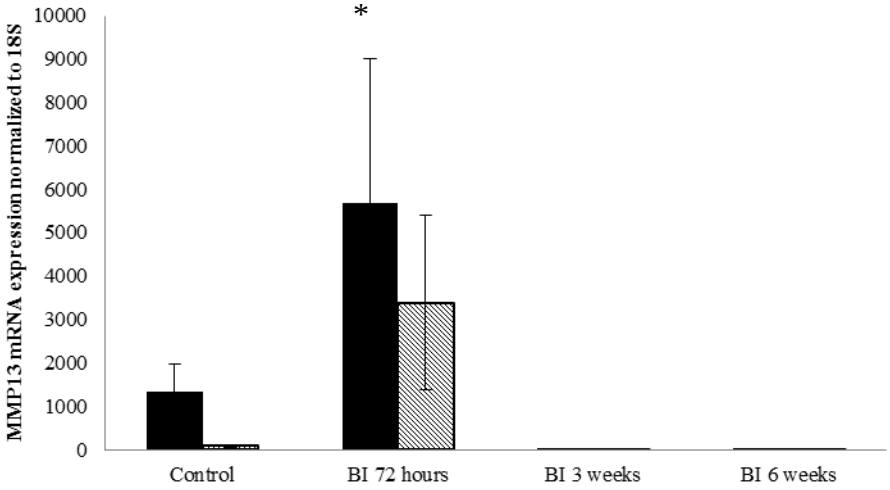

Histological Score
0
1
2
3
4
5
6
*
**
Ctl
Sham 3 weeks
Sham 6 weeks
Sham 9 weeks
BI 72 hours
BI 3 weeks
BI 6 weeks
BI 9 weeks
BI 52 weeks

5.3. Figure 3 Cartilage qPCR

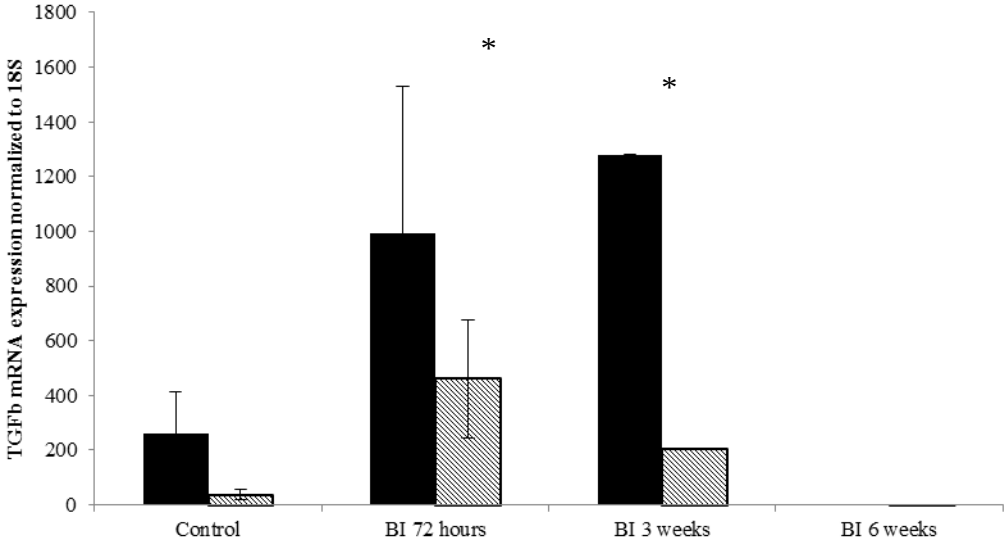
Figure 3. a) MMP13 mRNA expression in tibial plateau cartilage (pooled medial and lateral) of normal Control animals, and experimental animals 72 hours, 3 weeks and 6 weeks post-surgery.

b) Graph of TGFβ mRNA expression in tibial plateau cartilage (pooled medial and lateral) of normal Control animals, and experimental animals 72 hours, 3 weeks and 6 weeks post-surgery. Surgical limbs are the solid black boxes, and the contralateral limbs are the hashed boxes. mRNA was normalized to 18S. *= significantly different than control animals $p<0.05$.

a)


MMP13 mRNA expression normalized to 18S
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
*
Control
BI 72 hours
BI 3 weeks
BI 6 weeks

b)


TGFb mRNA expression normalized to 18S
1800
1600
1400
1200
1000
800
600
400
200
0
*
*
Control
BI 72 hours
BI 3 weeks
BI 6 weeks

5.4. Figure 4 Synovial qPCR

Figure 4. Synovial mRNA. a) IL-1β expression was significantly higher 72 hours post-surgery and in the surgical limb compared to the contralateral limb.

b) ILRa was significantly higher 72 hours post-surgery and was significantly higher in the surgical limb compared to the contralateral limb.

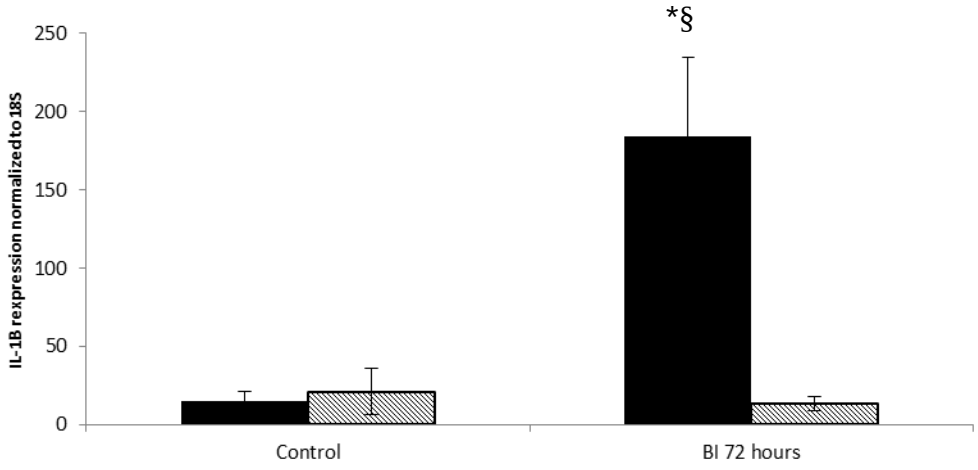
c) IL-6 was significantly higher 72 hours post-surgery and was significantly higher in the surgical limb compared to the contralateral limb.

d) IL-8 was significantly higher 72 hours post-surgery and was significantly higher in the surgical limb compared to the contralateral limb.

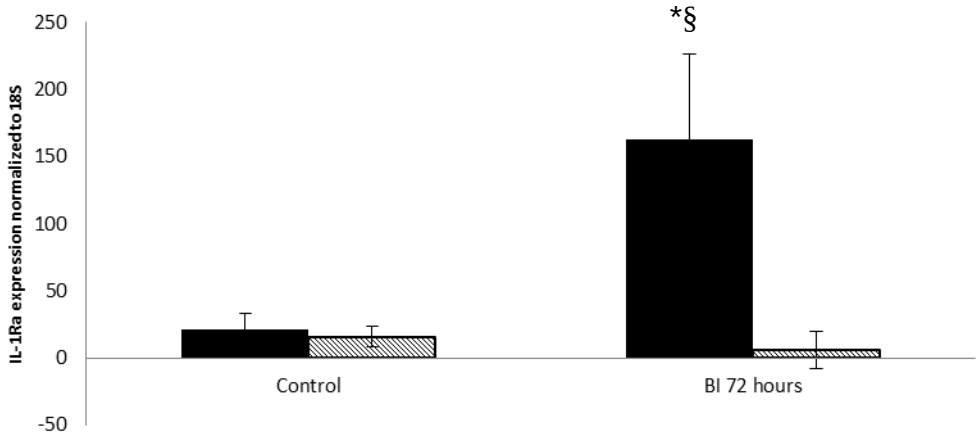
e) TNFα was not different from normal controls.

Surgical limbs are the solid black boxes, and the contralateral limbs are the hashed boxes.* =significant difference from Control $p<0.01$; §= significant difference from contralateral limb $p<0.05$. Note the 3 week and 6 week experimental animals (not shown) were not significantly different from normal Controls.

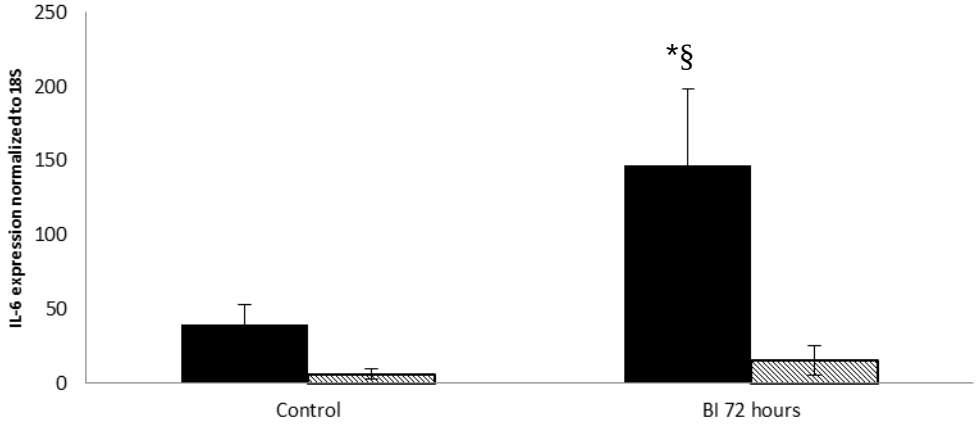
a)


*§
250
200
150
100
50
0
IL-1B rexpression normalized to 18S
Control
BI 72 hours

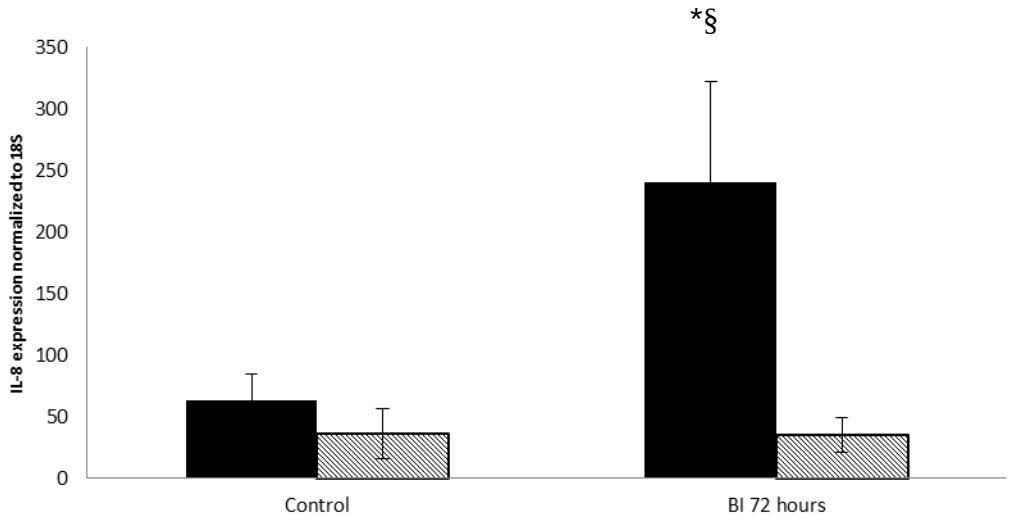
b)


*§
250
200
150
100
50
0
-50
IL-1Ra expression normalized to 18S
Control
BI 72 hours

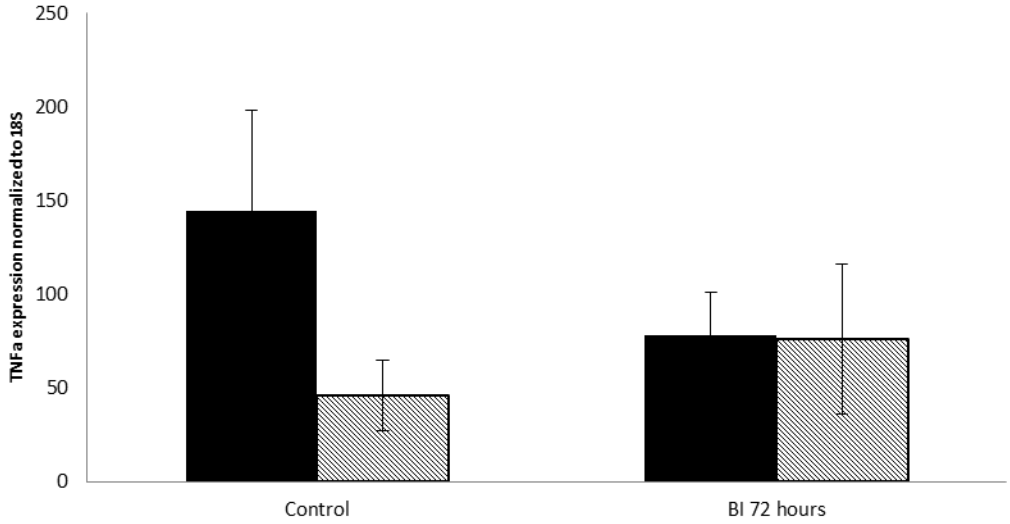
c)


*§
250
200
150
100
50
0
IL-6 expression normalized to 18S
Control
BI 72 hours

d)

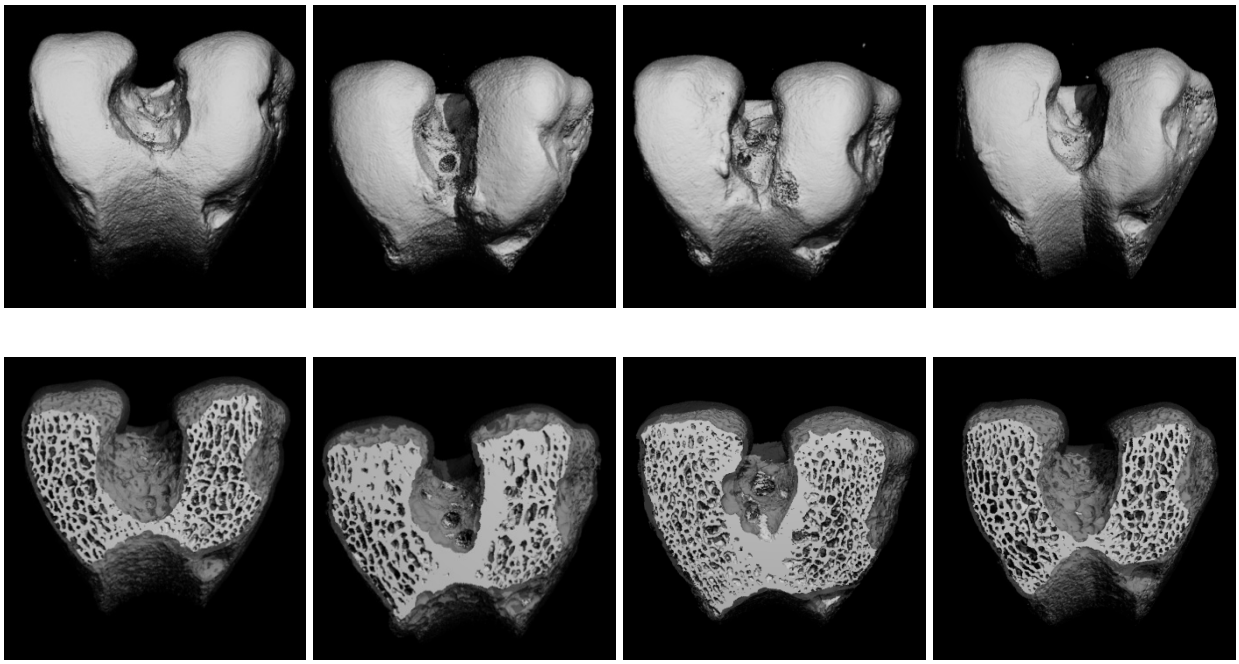

*§
350
300
250
200
150
100
50
0
IL-8 expression normalized to 18S
Control
BI 72 hours

e)


250
200
150
100
50
0
TNFa expression normalized to 18S
Control
BI 72 hours

5.5. Figure 5 Micro-CT

Figure 5. Healing of drill hole at time of euthanasia. µCT scans of the distal end of the femur and cross section of the analyzed region (cortical bone in dark grey). From left to right of Ctl, 3 weeks post-surgery, 9 weeks post-surgery, and 1 year post-surgery. Early after drilling (3w, 6w and 9w) there were significantly more Trabecular number ($p = 0.002$) post-drilling, significantly less Trabecular spacing ($p = 0.0009$) post-drilling and significantly higher connectivity density ($p = 0.008$) post-drilling compared to non-surgical controls.



7. DISCUSSION AND CONCLUSION

This study is the first to create a surgical model of PTOA in which the joint was not destabilized. While animals may have off-loaded the surgical side initially due to pain (not measured) I suspect that there was no significant change to joint loading. Animals appeared to return to normal activity within hours of surgery. I created a model in which intra-articular bone tunnels not involving any joint structures that would cause dysfunction, elicited early and persisting synovial changes and resulted in progressive gross and histological cartilage damage. Vaseenon *et al.* (322) showed global joint damage after an osteochondral defect; however, unlike my model their injury was much larger and affected load bearing areas of the joint which would result in changed joint mechanics. There has been considerable research on the functional and biological role of different models of OA; however, none of these models has been able to separate the pathogenic effects of injury-induced inflammation and biomechanics. I hypothesized that drilling holes into the non-load bearing bone within the joint would induce synovial inflammation, and more importantly, secondary cartilage damage as compared with sham joint invasion and normal animals. My results supported this hypothesis since after drill surgery, animals had significantly more cartilage changes and damage compared to all other animal groups (Ctl and Shams). My gross observations and histological scores were consistent with other rabbit models of cartilage damage and PTOA (323, 324), suggesting that bone tunnels did induce an incremental injury. I was clearly able to show that drilling into bone causes early catabolic changes and predisposes the joint to progressive cartilage damage as seen grossly and by significantly higher Mankin scores progressing from 72h up to 52w. It also caused gross bone changes (osteophytes) along with both synovial changes (significantly higher inflammatory expression at 72h; with significantly more cells at 9w and 52w). It was also evident in this model that arthrotomy alone, while not as severe as drilling, also led to mild cartilage damage. It is

likely that joint access alone is causing an inflammatory response leading to some cartilage damage and disease progression.

In addition to signs of cartilage damage in this model, I also showed that there was an early up-regulation in expression of MMP13 in the tibial plateau cartilage. MMP13 plays a central role in cartilage degeneration seen in both humans and in animal models. MMP13 cleaves collagen II, and targets aggrecan, collagen type IV and IX, osteonectin and perlican in cartilage (325). Investigations of cartilage from patients with OA have shown cloning of MMP13 from RNA isolated from human chondrocytes (326), and high levels of MMP13 expression (327). TGFβ has been identified as a potential mechanism in the development of OA(328), and is integral to cartilage maintenance. It's down-regulation has been shown to cause OA like changes (329). I found an up-regulation of TGFβ early post-surgery; there have been several mouse studies showing that up-regulation of TGFβ induces synovial fibrosis and osteophyte formation (330, 331). Perhaps the disruption of bone in my model causes changes in bone morphogenetic protein, part of the TGF family and leading to an up-regulation of TGFβ. It is also possible that TGFβ is up-regulated in my model to compensate for the up-regulation of MMP13, as one of the roles of TGFβ is to counteract MMP13 (332). I was unable to show these markers were up-regulated beyond 3w (TGFβ) or 72h (MMP13). I expected the inflammatory milieu to be up-regulated for a sustained period contributing to increased MMPs and catabolism. It is possible that since I am measuring expression as opposed to protein levels, protein levels may be higher and withstanding degradation in the joint. Another possibility is that other markers of cartilage degradation which I did not investigate are active later on.

While I found increased cell numbers in the synovium at later time points, I was unable to show up-regulation of inflammatory marker expression past 72 hours post-surgery. The fat pad is involved in intra-articular inflammation in OA with up-regulation of inflammatory genes

(333, 334). One consideration is that the synovium initiates early inflammation, while the fat pad plays a role in maintaining low grade inflammation in the osteoarthritic joint (unpublished data). Therefore it is possible that the cells in the synovium early after surgery are producing inflammatory markers in higher levels while later those cells are proliferating, or there is cell recruitment to produce other mediators of OA. In future studies I plan to investigate this phenomenon. Synovium from experimental animals were different from shams and were associated with significant cartilage changes. While cause-and-effect cannot be demonstrated, the association suggests increased synovial inflammation may be implicated in cartilage damage that was seen in this model.

Based on my observation of early inflammatory markers and on-going synovial changes and the known role(s) of synovitis in arthritis, I propose three potential mechanisms by which drill holes could induce synovitis and thus secondary cartilage damage: blood in the joint leading to synovitis, bone marrow elements in the joint leading to synovitis, and a secondary immune response from marrow elements in the joint.

Intra-articular fractures and bleeding are associated with OA development (335, 336) and repeated bleeds, as seen in haemophilia (337) lead to severe joint damage. It is possible that even a single episode of bleeding in the rabbit joint might contribute to synovial inflammation; fibrin can play a pro-inflammatory role and synovial exposure to blood and hemopoietic cells has been shown to lead to chronic synovitis (338). It has also been demonstrated that short term cartilage exposure to blood *in vitro* results in the inability of chondrocytes to restore proteoglycan synthesis due to chondrocyte apoptosis. This mechanism may lead to joint damage and OA (339). While blood exposure may be one factor contributing to synovial inflammation and cartilage damage, it is highly unlikely that it is the only mechanism, as an arthrotomy alone did not lead to as much cartilage damage as was seen after drilling.

Bone marrow is known to have an immunogenic capacity (340), and contains a variety of stem cells with the capacity to differentiate into pro-inflammatory cells. These cells could affect various structures in the joint. I confirmed that the bone tunnels filled slowly, allowing many weeks for potential leakage of marrow elements into the joint. Leaking stromal cells have the capacity to produce pro-inflammatory proteins and could be responsible for inflammation and subsequent cartilage damage.

Finally, I suggest there may also be secondary immune responses to marrow elements in the joint. The joint functions as an organ, immunologically isolated from the rest of the body by a fibrous capsule, sealed in early embryonic development. Hence, the body may perceive marrow elements as foreign, inducing an immune response. This secondary immune response could both stimulate synovial inflammation and attack articular cartilage through recruitment of phagocytic immune cells.

In conclusion, I have been able to develop a new, surgically relevant model of bone injury-induced PTOA that alters the joint environment without grossly changing joint mechanics. The mechanisms of the secondary cartilage damage could be due to a number of changes in the joint environment, but it seems likely that the final pathway in this model involves inflammatory changes of synovium. While these changes may develop more quickly in the more reactive rabbit than in humans, this model provides a new avenue for investigating the potential pathways of post-traumatic OA.

Chapter 3. DEXAMETHASONE INHIBITS INFLAMMATION AND CARTILAGE DAMAGE IN THIS MODEL OF PTOA

1. ABSTRACT

Corticosteroids are used in musculoskeletal diseases; and offer patient relief. Injections of corticosteroids are recommended for management of osteoarthritis (OA). Current data have shown the role of corticosteroids in ameliorating pain. I hypothesized that repeated intra-articular injections of high dose dexamethasone would protect the cartilage from damage in a post-traumatic model of OA. Eighteen female New Zealand White rabbits were used. Twelve underwent surgery to induce OA; six of them received intraarticular injections of dexamethasone every 3 days for 3 weeks. The other six rabbits served as operated controls. Six additional rabbits served as non-operated controls. All animals were euthanized 3 weeks post-surgery. Knees were assessed grossly. Cartilage, synovium, and fat pad were assessed histologically. Synovium and fat pad were analyzed with qPCR and Western blots. Surgical controls had cartilage damage which was supressed with dexamethasone. Dexamethasone significantly decreased synovial expression of interleukin-1b and collagen I, and a trend to decrease synovial matrix metalloproteinase3 expression. There were also significantly lower levels of interleukin-1b protein with dexamethasone treatment. Dexamethasone significantly decreased fat pad expression of matrix metalloproteinase13, basic fibroblast growth factor, and interleukin8, and a trend to decrease matrix metalloproteinase3 and transforming growth factorb expression. Dexamethasone decreased joint inflammation and joint tissue degradation and was chondroprotective in this unique model of PTOA.

2. INTRODUCTION

Osteoarthritis (OA) is one of the leading causes of disability worldwide (307), affecting over 4.5 million Canadians (341). OA commonly occurs after joint injuries. Anterior Cruciate Ligament (ACL) injuries and other knee injuries are the most common cause of secondary OA. While there have been many surgical advances in the treatment of knee injuries, there seems to be little improvement in the natural progression of OA (342, 343). This has stimulated a debate amongst researchers regarding the mechanisms driving OA; resulting in a theory that inflammation is important in the pathogenesis of OA.

Numerous studies have investigated the efficacy of intra-articular injections of corticosteroids in the management of OA symptoms. In a review of 28 clinical trials Ringdahl *et al.* (344) found that corticosteroid injections reduced pain in the short-term compared to placebo; however, there was no evidence of long term benefits. These studies also showed no adverse effects with short-term corticosteroid use.

Dexamethasone is a potent corticosteroid that has a high binding affinity to intracellular glucocorticoid receptors enhancing transcription of anti-inflammatory cytokines such as interleukin 1 receptor antagonist (IL-1Ra) and interleukin 10 (IL-10) (345), and repressing transcription of pro-inflammatory cytokines, such as interleukin 1β (IL-1β), interleukin 6 (IL-6), cyclooxygenase 2 (COX2), and tumor necrosis factor α (TNFα) (345, 346). Dexamethasone also has an effect on cartilage potentiating chondrocyte differentiation and proteoglycan synthesis *in vitro (347)*, and reduced glycosaminoglycan loss in cartilage injury *in vitro* (348). Dexamethasone enhances progenitor cells to differentiate into chondrogenic cells and synthesize proteoglycans.

As dexamethasone is a potent anti-inflammatory and it is potentially chondroprotective *in vivo*, I sought to investigate its role in inhibiting inflammation and cartilage damage in a model

of post-traumatic OA (PTOA). I hypothesized that frequent high dose intra-articular injections of dexamethasone would decrease inflammation post-injury and prevent cartilage changes in a 'non-mechanical' model of PTOA.

3. MATERIALS AND METHODS

3.1. Animal model

Eighteen mature female New Zealand White rabbits were used to investigate the role of dexamethasone as describes by Huebner *et al. (349)*. Twelve rabbits underwent drill hole bone-injury surgery to induce PTOA as noted above; six of them received intra-articular dexamethasone injections (called DEX) and the other six rabbits served as operated, surgical controls. The remaining six rabbits served as non-operated, untreated controls. All animal work was approved by the University of Calgary Animal Ethics Committee in accordance with the Canadian Council on Animal Care (M08085).

3.1.1. Non-mechanical PTOA surgery

Twelve rabbits underwent surgery of the right hind limb to induce bone injury using intra-articular drilling (349), See Chapter 2 for details. Animals returned to normal cage activity immediately after surgery and were euthanized 3 weeks post-surgery.

3.1.2. Dexamethasone injections

Six of the twelve PTOA surgery animals were randomly allocated to the dexamethasone treated group (DEX). Dexamethasone (Dexamethasone 5, Vetoquinol N.-A. Inc. Lavaltrie, QC) was injected intra-articular in the right knee joint at a dose of 0.5mg/kg. This dose was chosen based on previous studies in rabbits and rats (350-352) in order to fully inhibit inflammation. The initial dose was given at surgery, a second dose was given 6 hours post-operatively, then injections were given every third day until the time of euthanasia at 3 weeks.

Gross morphology of the joint was assessed qualitatively for osteophytes, cartilage lesions, discolouration and fibrillation by two experienced observers. At the time of euthanasia the fat pad, then articular cartilage and synovium were collected. Synovium was sampled from the femoral groove and suprapatellar region. Cartilage samples were taken from the centre of six

different locations in the joint: femoral groove, patella, lateral and medial femoral condyle, and lateral and medial tibial plateau. Samples were flash frozen for histology and molecular assays.

3.2. Histology

3.2.1. Cartilage

Cartilage was embedded in Tissue-Tek® O.C.T.™ (Sakura Finetek USA Inc., Torrance, CA), flash frozen in isopentane on dry ice, and cryosectioned (5μm). Slides were stained with Safranin-O/Fast-green and graded blind by one observer twice with a modified Mankin score (349, 353). Six sections from each area of each joint were analyzed. As no differences were found across different areas of the joint, they were summed.

3.2.2. Synovium

The synovium was embedded in Tissue-Tek® O.C.T., flash frozen in isopentane on dry ice, and cryosectioned (10μm). Slides were stained with Hematoxylin and Eosin. Synovial membrane thickness was analyzed with Image J software (National Institute of Health, Bethesda, MD, USA) taking an average thickness of three sections for each animal.

3.2.3. Immunohistochemistry of Fat Pad

The embedded in Tissue-Tek® O.C.T.™, flash frozen in isopentane on dry ice, and cryosectioned (10μm). Slides probed with one of six primary antibodies: IL-8 (1:1500; mouse monoclonal ab34100; Abcam Inc., Toronto, ON), IL-1Ra (1:2000; goat polyclonal sc-8481; Santa Cruz; Santa Cruz, CA), IL-1β (1:2000; goat polyclonal sc-1252; Santa Cruz, Santa Cruz, CA), TGFβ (1:2000; mouse monoclonal ab27969; Abcam Inc., Toronto, ON), bFGF (1:2000; goat polyclonal sc-1360; Santa Cruz; Santa Cruz; CA), and macrophage marker MAC387(1:1000; mouse monoclonal MAC387 ab22506; Abcam Inc., Toronto, ON), and then stained with one of two HRP-conjugated secondary antibodies: Goat anti-mouse HRP (1:200; mouse IgG H/L chain NBP1-74800; Novus Bio, Oakville, ON) and Donkey anti-goat HRP

(1:250; goat IgG H/L chain NBP1-75130; Novus Bio, Oakville, ON). All slides were then stained with diaminobenzidine (SigmaFast DAB with Metal Enhancer; Sigma-Aldrich Co., St. Louis, MO) and analyzed with Axiophot 2 (Carl Zeiss, Thornwood NY). The sections were scored blind on a scale of 0 to 3 based on the level of stain.

3.3. Real-time reverse transcriptase polymerase chain reactions (qPCR)

The synovium and fat pad of non-operated controls, surgical controls and DEX animals were examined for expression of inflammatory markers: IL-1β, IL-8, IL-6, and TNFα, for degradative markers: matrix metalloproteinases MMP3 and MMP13, for growth factor markers critical in cartilage maintenance: transforming growth factor β (TGFβ) and fibroblast growth factor (bFGF), and for the connective tissue marker collagen I. All samples were normalized to 18S mRNA. Expressions were analyzed using qPCR as described by Reno *et al.* (317). See appendix 3 Table 2 for primer sequences.

3.4. Western Blots

The fat pad and synovium of non-operated controls, surgical controls and DEX animals's right hind limbs were examined for protein levels (n=4). Protein extraction, loading and separation were done as previously described by Huebner *et al.*(349).

3.4.1. Protein extraction

Protein was isolated from the Trizol layer following the manufacturer's directions (Qiagen Sciences, Germantown, Maryland) during RNA extraction, described above. The pellet was re-suspended in Tissue Protein Extraction Reagent (T-PER; Pierce, Rockford, Illinois) and EDTA-free halt protease inhibitor cocktail, (Pierce, Rockford, Illinois).

3.4.2. SDS-PAGE and Western blot

Loading samples were prepared and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using NuPAGE 4-12% Bis-Tris gel in 1X MES

SDS running buffer at 110V for one and a half hours. Proteins separated on the gel were then transferred to a polyvinylidene fluoride (PVDF) membrane at 20V for one and a half hours. Membranes were incubated with antibodies directed against bFGF (1:750; C-18 sc1360 Santa Cruz Biotecnologies Inc, Santa Cruz, California) or IL-1Ra (1:750; Q-19 sc8481 Santa Cruz Biotecnologies Inc, Santa Cruz, California) or IL-1β (1:1000; R-20 sc1252 Santa Cruz Biotecnologies Inc, Santa Cruz, California) or IL-6 (1:750; M-19 sc1265 Santa Cruz Biotecnologies Inc, Santa Cruz, California) or IL-8 (1:1000; 8M6 ab34100 Abcam, Cambridge, United Kingdom) or TGFβ (1:1000; TB21 ab26969 Abcam, Cambridge, United Kingdom) at 4^0C overnight. Membranes were incubated for one hour at room temperature in an HRP-conjugated secondary antibody: bFGF, IL-1Ra, IL-1β and IL-6 were incubated with anti-goat IgG (1:5000; NBP1-74800 Novus Biotechnology, Oakville, Ontario), GAPDH, TGFβ and IL-8 were incubated with anti-mouse IgG (1:5000; NBP1-75130 Novus Biotechnology, Oakville, Ontario). The membranes were developed using Novex chemiluminescent substrates (Invitrogen, Burlington, Ontario) and electro-chemiluminescent detection kit (Amersham Biosciences; Buckinghamshire, United Kingdom) and the blots were quantified by densitometry. Antibodies directed against GAPDH (1:2000; GA1R ab125247 Abcam, Cambridge, United Kingdom) were used as a loading control.

3.5. Statistical Analysis

Statistical analysis of cartilage Mankin scores was performed with ANCOVA with Bonferroni post-hoc (CI=95%). Scores were also compared between joint locations and subscores with ANCOVA with Bonferroni post-hoc (CI-95%). Synovial membrane thickness was analyzed with a UNIANOVA with LSD post-hoc (mean difference (MD) + CI=95%). Immunohistochemistry data were analyzed with a MANOVA with LSD post-hoc. All qPCR and Western blot data were analyzed using a MANOVA with Bonferroni post-hoc and confirmed

with a Kruskal-Wallis test with Tukey HSD post-hock. All tests used unequal variance assumptions and non-parametric statistics were performed to confirm results to account for size differences between groups and variability within groups. A $p<0.05$ was considered significant (SPSS; IBM, Armonk, NY).

4. RESULTS

During the course of the study non-operated and surgical controls appeared healthy, were active in their cages and maintained a normal diet and fluid intake. All animals including DEX animals regained baseline activity moving around their cages within 6 hours of surgery (not quantified) and they continued to be mobile over the 3 week course of this study. DEX animals ate and drank more than other animals (not quantified) and lost weight despite increasing their caloric intake. DEX animals lost an average of 11.25% body weight (range 2% to 28.89%), while the other groups had no gain or loss of weight. In addition to weight loss, changes were noted upon gross inspection of the organs of the DEX animals during necropsy. The DEX hearts were relatively fibrous with heterogeneous pigment changes, the lungs appeared more edematous with fibrotic and necrotic areas, the livers had some areas of necrosis and were friable, and the kidneys were yellowed, nephrotic tissue was heterogeneous in appearance and their internal architecture was distorted.

4.1. Cartilage and Synovium – Gross and histology

Signs of gross surface damage were apparent in the surgical control animals, showing some fibrillation and discolouration. The DEX animals had no signs of cartilage damage; their articular cartilage was indistinguishable from non-operated controls.

The gross findings were consistent with the microscopic changes seen with histology. Surgical control animals had abnormal cartilage with chondrocyte cloning, surface damage and loss of safranin-O staining. Non-operated controls right and left cartilage had significantly lower Mankin scores than operated controls left knees ($p=0.036$; MD = 9.31; 95% CI = 0.63 to 17.98) and right knees ($p<0.0001$; MD = 28.39; 95% CI = 19.72 to 37.06). Non-operated controls Mankin scores did not differ from DEX animal scores ($p>0.05$). Operated control's left limbs had significantly higher Mankin scores compared to DEX right ($p=0.034$; MD = 9.39; 95% CI =

0.72 to 18.09) and left (p=0.013; MD = 11.06; 95% CI = 2.38 to 19.73) limb scores. Their right limbs also had significantly higher Mankin scores compared to DEX right (p<0.001; MD = 28.47; 95% CI = 19.80 to 37.15) and left (p<0.0001; MD = 30.14; 95% CI = 21.47 to 38.81) limb scores (Figure 1). Operated controls right limb had significantly higher Mankin scores than their non-surgical left limb (p<0.0001; MD = 19.08; 95% CI = 10.41 to 27.56). There were no differences between right and left limbs within non-operated control and DEX groups (p>0.05). There were no differences between subscores nor joint locations (p>0.05), therefore the Mankin scores above were pooled and average for a total joint score for each animal.

The synovium did not differ in thickness among the operated groups (p>0.05), and there was no difference in cell infiltrate present (p>0.05).

4.2. Immunohistochemistry

TGFβ, bFGF and IL-1Ra were not significantly different in level of positive stain between groups (p>0.05). The right fat pad of surgical control had significantly more positive stain for IL-8 (p=0.004; MD = 1.40; 95% CI = 0.50 to 1.30), IL-1β (p=0.002; MD = 1.20; 95% CI = 0.53 to 1.87) and MAC387 (p=0.005; MD = 1.33; 95% CI = 0.32 to 2.35) than non-operated controls. Although not statistically significant, DEX samples had lower levels than surgical controls. These makers did not differ between DEX and control groups (p>0.05).

4.3. qPCR

mRNA expression of synovial IL-1β was significantly greater in the right limb of surgical controls than the right limb of DEX animals (p=0.015; MD = 12.39; 95% CI = 2.56 to 22.26) (Figure 2a). Collagen I expression was also significantly higher in surgical control synovium compared to DEX synovium (p=0.002; MD = 12.28; 95% CI = 4.73 to 19.84) (Figure 2b). mRNA expression of synovial MMP3, although not statistically significant, was lower in DEX

compared with surgical controls (p>0.05) (Figure 2c). There were no other differences in mRNA levels in the synovium between groups.

In the fat pad, the surgical controls had higher expression of MMP13 and bFGF compared to non-operated controls (p=0.014; MD = 0.50; 95% CI = 0.11 to 0.90 and p=0.01; MD = 0.50; 95% CI = 0.10 to 0.89 respectively) and DEX (p=0.012; MD = 0.58; 95% CI = 0.13 to 1.02 and p=0.006; MD = 0.01; 95% CI = 0.10 to 0.89 respectively) (Figure 3a and b). Unexpectedly, the DEX right limb fat pad IL-8 expression was significantly higher than non-operated controls (p=0.006; MD = 0.13; 95% CI = 0.02 to 0.23), surgical controls (p=0.006; MD = 0.13; 95% CI = 0.02 to 0.23), and DEX left limb (p=0.007; MD = 0.13; 95% CI = 0.02 to 0.23) (Figure 3c). While not significant, DEX had lower mean expression of MMP3 and TGFβ compared to surgical controls. There were no other differences in mRNA levels in the fat pad between groups.

4.4. Western Blots

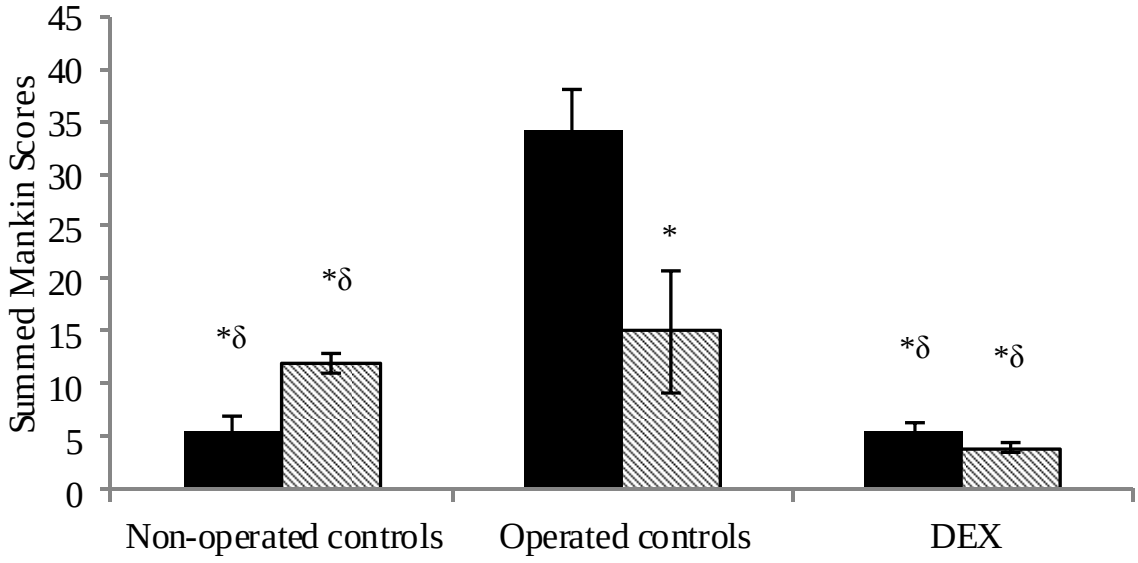
bFGF protein levels in the synovium were lower after dexamethasone treatment than 3 week surgical controls (NSD p>0.05). There were significantly lower protein levels of IL-1β in the synovium from the right leg in DEX compared to surgical controls (p=0.027; MD=0.01; 95% CI = -0.02 to 0.04). However, there were no differences in the levels of IL-6, IL-8 or TGFβ protein with dexamethasone treatment compared to non-operated controls or surgical controls (p>0.05). Figure 4a-b.

There were no significant differences in protein levels in the fat pad due to small sample sizes and large variability; however, there were trends in the fat pad IL-1β protein levels which were lower in the DEX animals than surgical controls, as was the ratio of IL-1β/IL-1Ra (Figure 4c-d). There were no differences in the levels of bFGF, IL-1Ra, IL-6, IL-8, or TGFβ between groups (p>0.05).

5. FIGURES

5.1. Figure 1 Cartilage histology

Figure 1: Summed mean Mankin scores of the articular cartilage (n=6/group). The solid boxes = right limb, hatched boxes = left limb. Operated controls had significantly higher Mankin scores than the non-operated controls and DEX animals ($p>0.05$). Error bars represent standard deviation. * = significant difference from right operated controls; δ = significant difference from left operated controls.


Summed Mankin Scores
45
40
35
30
25
20
15
10
5
0
*δ
*δ
*
*δ
*δ
Non-operated controls
Operated controls
DEX

5.2. Figure 2 Synovial qPCR

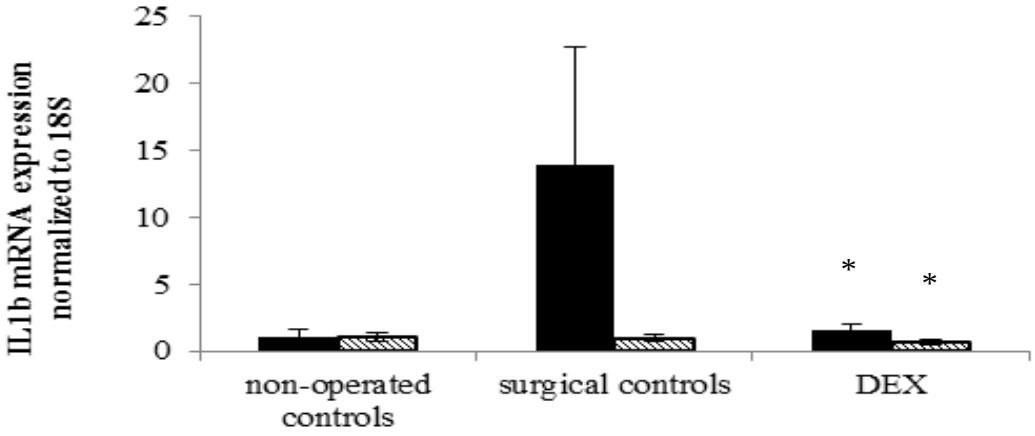
Figure 2. Synovial mRNA expression of IL-1B. Solid = mean of right limb; hatched boxes = mean of left limb. (n=6/group) Error bars represent standard deviations. a) DEX means of right and left had significantly lower levels of IL-1β expression.

b) DEX means of right and left had significantly lower levels of collagen I expression.

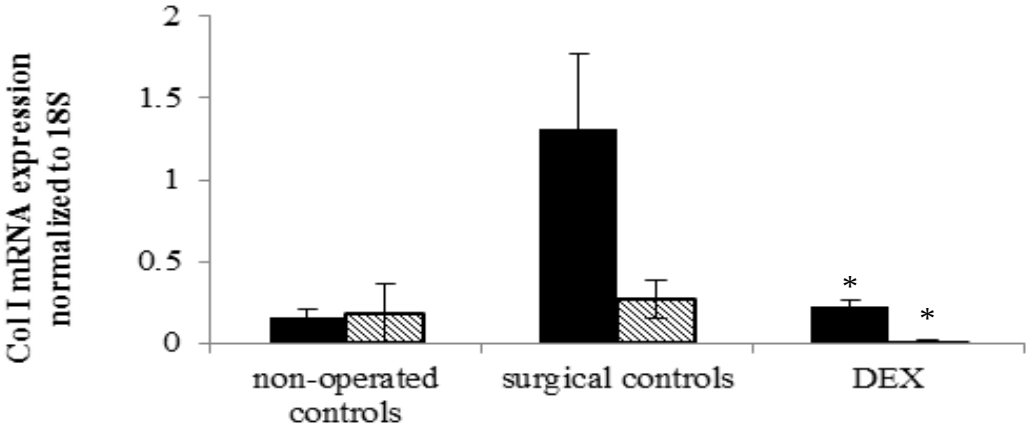
c) While not significant DEX synvovium had lower mean levels of MMP3 expression than surgical controls.

* = significant difference from right surgical controls $p<0.05$.

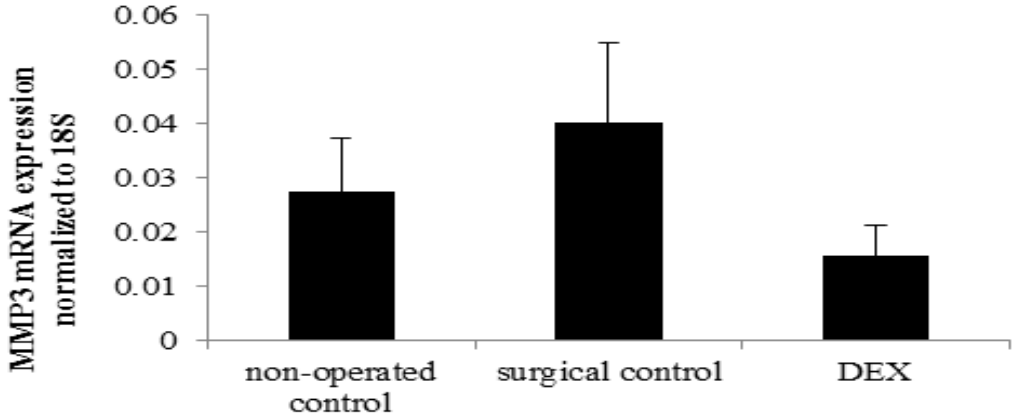
a)


IL1b mRNA expression normalized to 18S
25
20
15
10
5
0
*
*
non-operated controls
surgical controls
DEX

b)


Col I mRNA expression normalized to 18S
2
1.5
1
0.5
0
*
*
non-operated controls
surgical controls
DEX

c)


MMP3 mRNA expression normalized to 18S
0.06
0.05
0.04
0.03
0.02
0.01
0
non-operated control
surgical control
DEX

5.3. Figure 3 Fat pad qPCR

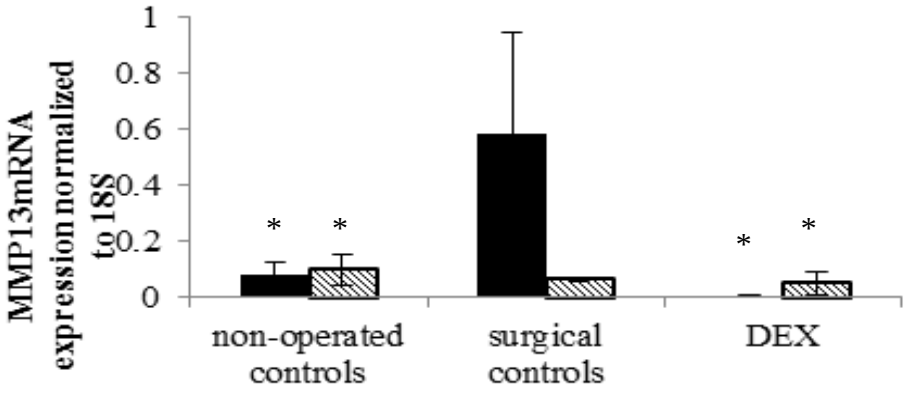
Figure 3. Fat pad mRNA expression. (n=6/group) Error bars represent standard deviation. Solid boxes = right, hatched boxes = left. a) surgical control right limbs had significantly higher mean MMP13 expression.

b) Surgical control right limbs showing significantly higher mean bFGF expression.

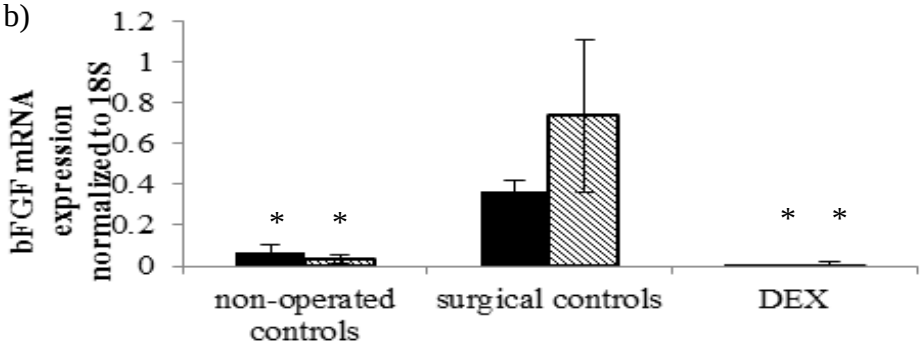
c) DEX right limbs had significantly higher mean IL-1β expression.

* = $p<0.05$ from right surgical controls; # = $p<0.05$ from right DEX.

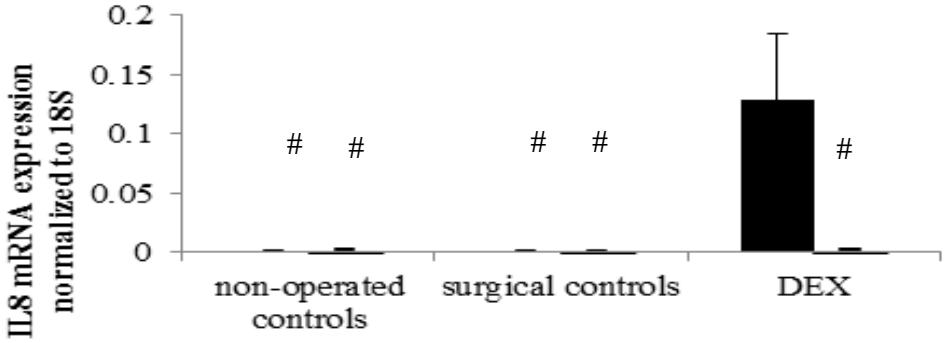
a)


MMP13mRNA expression normalized to 18S
1
0.8
0.6
0.4
0.2
0
*
*
*
*
non-operated controls
surgical controls
DEX

b)


bFGF mRNA expression normalized to 18S
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
*
non-operated controls
surgical controls
DEX

c)


IL8 mRNA expression normalized to 18S
0.2
0.15
0.1
0.05
0
#
#
#
#
#
non-operated controls
surgical controls
DEX

5.4. Figure 4 Synovial and Fat pad protein levels

Figure 4. Synovial and Fat pad protein expression of right hind limbs. (n= 4/group) Error bars represent standard deviation. a) DEX right synovium had a trend of decreased bFGF to surgical controls ($p>0.05$).

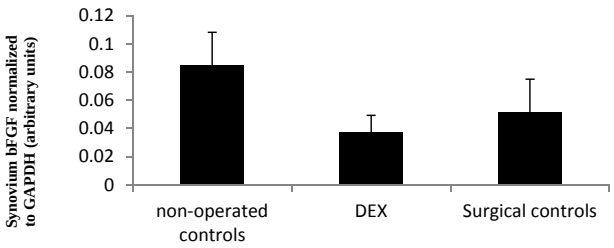
b) DEX right synovium had significantly lower levels of IL-1β protein than surgical controls.

c) DEX right fat pad had a trend of decreased IL-1b to surgical controls ($p>0.05$).

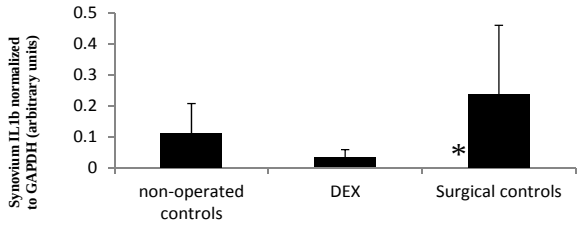
d) DEX right fat pad had a lower ratio of IL-1β/IL-1Ra protein levels than surgical controls.

* = $p<0.05$ from surgical controls.

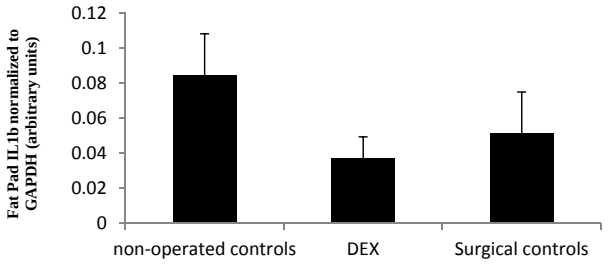
a)


Synovium bFGF normalized to GAPDH (arbitrary units)
0.12
0.1
0.08
0.06
0.04
0.02
0
non-operated controls
DEX
Surgical controls

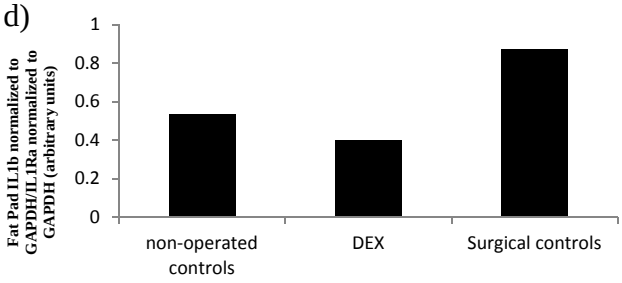
b)


Synovium IL1b normalized to GAPDH (arbitrary units)
0.5
0.4
0.3
0.2
0.1
0
*
non-operated controls
DEX
Surgical controls

c)


Fat Pad IL1b normalized to GAPDH (arbitrary units)
0.12
0.1
0.08
0.06
0.04
0.02
0
non-operated controls
DEX
Surgical controls

d)


Fat Pad IL1b normalized to GAPDH/IL1Ra normalized to GAPDH (arbitrary units)
1
0.8
0.6
0.4
0.2
0
non-operated controls
DEX
Surgical controls

7. DISCUSSION AND CONCLUSIONS

Corticosteroids, such as prednisone and dexamethasone have been used in the management of osteoarthritis for over four decades (354-356). They are part of the practice guidelines for treatment of OA (209) and have been proven to be safe and efficacious for pain relief and functional improvements in OA patients both short term (357, 358) and long term (359-361). Corticosteroids are also widely used for treatment in inflammatory conditions, such as rheumatoid arthritis, lupus, and inflammatory bowel disease. Therefore, much work has been done on their role in modifying inflammation in OA. The majority of the work, however, has been done either in *in vitro* models, or in models inducing joint inflammation with chemical mediators.

My study is the first to demonstrate a chondroprotective effect of dexamethasone in a non-mechanical surgical model of PTOA (349). Dexamethasone treatment has been previously shown to have a chondroprotective effect *in vitro* by completely blocking TNFα induced GAG loss (348). I was able to alter inflammatory cytokines and gene expression in synovium and prevent both gross and histological cartilage damage caused after surgery with repeated high dose intra-articular dexamethasone injections post-injury. I hypothesized that a short-course of repeated high dose intra-articular injections of dexamethasone would diminish joint inflammation and thus change joint tissue catabolism and anabolism preventing post-traumatic cartilage damage. My results support this hypothesis since treatment with dexamethasone after surgery significantly reduced the amount of cartilage damage compared to the surgical control animals. I was clearly able to show that 3 weeks of dexamethasone treatments elicited less gross joint damage and significantly lowered cartilage damage as seen by Mankin scores. This is consistent with previous work showing that treatment with dexamethasone in TNFα-induced OA *in vivo* (362), mechanical injury + TNFα + IL-6 induced OA *in vitro* (348) and IL-6 induced OA

in vitro (363) prevents glycosaminoglycan loss, prevents aggrecan degradation and increases proteoglycan synthesis. Furthermore work by Livne *et al.* (364) demonstrated that dexamethasone stimulated chondrocyte proliferation and rejuvenated the chondrocyte population in the mandibular condyles of old mice. Despite some *in vitro* evidence that glucocorticoids might be chondrotoxic (365, 366), my work and other *in vivo* studies suggest that dexamethasone can regulate the catabolic effects of cartilage post-trauma.

In addition to chondroprotective effects of dexamethasone in this model, I also showed changes in synovial markers of inflammation, fibrosis, and degradation. Vento *et al.* (367) found that when treating synovial tissue cultured from OA patients with dexamethasone there was a reduction in synovial cell fibroblasts and morphological cell changes. I was unable to find any histological decrease in cell number with dexamethasone treatment compared to surgical controls. It is possible that 3 weeks is too late to see truly acute inflammatory proliferation from the injury and too late for remodelling of the membrane and therefore I may have simply missed the window to see these changes histologically. Despite no histological evidence of changes in inflammation, there was a significant decrease in the expression of synovial IL-1β expression and protein levels with dexamethasone. IL-1β is thought to be involved in the development of OA with an acute inflammatory event (368), and induces COX2 as well as activates MMPs. Dexamethasone suppresses IL-1β therefore decreasing COX2 in rheumatoid arthritis synovial tissue (369). It also inhibits chondrocyte MMP production in IL-1β stimulated cultures (370). While I found a down-regulation of synovial IL-1β, no other inflammatory cytokines were suppressed with dexamethasone. Synovial collagen I expression was suppressed by dexamethasone; I suspect that dexamethasone inhibited fibroblast proliferation that increases synovitis and fibrosis after injury preventing a destructive environment.

Synovial MMP3 is up-regulated in various models of OA (371, 372) and has been correlated to more severe radiographic OA (373). It degrades collagen type II, III, IV, IX and X, as well as proteoglycans, laminin, elastin and fibronectin all important in the integrity and architecture of the joint structures. While not significant, I found a trend for dexamethasone to decrease the expression of synovial MMP3 after inducing PTOA. No other MMPs had lower expression with treatment, despite the mechanism MMP3 has on activating other MMPs. It is possible that the levels of MMP3 were not high enough to activate the other synovial MMPs.

In addition to the role of synovium in OA several theories have emerged suggesting that the fat pad may play a role in OA. Obesity predisposes patients to OA, traditionally this was thought to be because of increased joint loading; however, new evidence suggest that part of the pathogenesis may be due to increased fat pad leading to increased joint inflammation (374, 375). Therefore I hypothesized that increased in inflammation of the fat pad would be present in a model of PTOA and that this would be suppressed by dexamethasone. This theory was supported by evidence showing an increase in IL-8 protein with immunohistochemistry in surgical controls. There was a non-significant decrease in IL-8 protein levels with dexamethasone. IL-8 is a potent chemokine which increases the inflammatory environment by attracting white blood cells and stimulating interleukin production and release. However, there was a significant increase in IL-8 expression of the right surgical limb in dexamethasone treated animals; I suspect this may be due to variability between animals due to the small sample size. The fat pad also had increased protein levels of MAC387 a macrophage marker and IL-1β in surgical controls, and while not significant due to the smaller sample size and variability, dexamethasone appeared to lower the levels of these proteins. Unfortunately due to a lack of protein in two of the animals all western blots were done with an n=4. There was also a decrease in the ratio of IL-1β/IL-1Ra in the fat

pad with dexamethasone. This suggests that the fat pad plays an important role maintaining joint inflammation and dexamethasone has the potential to suppress this.

In addition to changes in inflammation of the fat pad there was increased expression of degradative markers and growth factors with surgery that was suppressed with dexamethasone injections. bFGF and MMP13 were significantly higher post-surgery. MMP13 cleaves collagen II and degrades aggrecan, collagen IV and IX (325), while bFGF is a growth factor for angiogenesis secreted by adipocytes. Angiogenesis will increase blood into the joint, which leads to OA (376, 377). Dexamethasone suppressed expression of both bFGF and MMP13, therefore protecting the knee from developing OA. While not significant, dexamethasone also decreased MMP3 expression and TGFβ expression. Many studies have shown that the down-regulation of TGFβ contributes to OA; we previously demonstrated that TGFβ was up-regulated in PTOA and speculated that this was to compensate for the up-regulation of MMP13 (349). Su *et al.* (378) found that dexamethasone suppressed TGFβ in an *in vitro* model of OA by interfering with TGFβ signalling and inhibiting endogenous TGFβ.

At the dosages that were used in this study I found that dexamethasone caused weight loss and had systemic effects on several organ systems. The dosages used in this study were very high, and would not be used clinically; however, they were selected to prevent joint inflammation over the study course which proved effective. As I have been able to show that at these doses joint inflammation was prevented thereby protecting the knee it is important in future not only to assess lower, less frequent dosages (348), as well as longer term studies. It is of note that the animal groups were small; however, despite small sample sizes I was able to show significant differences.

In conclusion, my results demonstrated that administration of repeated high dose intra-articular injections of dexamethasone inhibits synovial inflammation and is chondroprotective in

a model of PTOA for up to three weeks *in vivo*. However, my results also showed that this dosage and treatment regime did have adverse systemic effects on the rabbits and thus would clearly not be advised for PTOA management in humans. It has nonetheless given us further insight into the role of inflammation in PTOA and provided new avenues for potential disease modifying treatments. As well as suggested that early corticosteroid injections after an injury may prevent later cartilage damage and joint degeneration. These results have also demonstrated that my model of PTOA causes joint inflammation resulting in cartilage damage, as dexamethasone is a potent anti-inflammatory agent.

Chapter 4. IL-1Ra PREVENTS SOME CARTILAGE DAMAGE IN PTOA

1. ABSTRACT

Osteoarthritis (OA) is a debilitating disease affecting millions of Canadians. A large proportion of these individuals developed OA after a joint injury. Inflammation post-injury is in part responsible for the development of OA. Interleukin (IL) 1β has been implicated as major component of inflammation after an injury and more potent than other inflammatory cytokines like tumor necrosis factor (TNF) α in stimulating matrix metalloproteinases (MMPs) and delaying cartilage repair. Anakinra, a recombinant IL-1Ra has been used for the treatment of rheumatoid arthritis (RA) and several studies have shown it to be chondroprotective in models of OA.

I therefore sought to inhibit inflammation and prevent cartilage damage in a new model of post-traumatic OA (PTOA) with IL-1Ra. I hypothesized that intraarticular injections of IL-1Ra similar to current clinical doses would prevent early inflammation and later cartilage damage in my model. In order to examine the effects of IL-1Ra, I treated twelve rabbits with Anakinra daily after surgery to their right hind limb. Non-operated and surgical controls were run concurrently. Animals were euthanized at 72 hours after surgery or 3 weeks after surgery. Joints were examined grossly and histologically. Fat pad, synovium and cartilage were assessed for expression and protein levels of inflammatory mediators, proteases and growth factors. IL-1Ra treatment resulted in partial protection of the cartilage, likely through basic fibroblast growth factor (bFGF) by increasing IL-1Ra in the fat pad and decreasing IL-1β in the synovium. IL-1β inhibition only partially prevented the development of PTOA, suggesting that future treatment targets must either be broader or upstream of IL-1β.

2. INTRODUCTION

Osteoarthritis (OA) is a disease affecting large numbers of North Americans (307, 341), involving the knees, hips, hand and spine predominantly. Approximately 12% of individuals suffering from OA are those who had a previous injury and developed post-traumatic OA (PTOA) (379). The original dogma was that the trauma itself led to joint instability and altered joint loading predisposing the joint to OA. However, evidence from anterior cruciate ligament (ACL) injuries show that despite normal post-repair/reconstruction biomechanics the knee still develops OA (380). A growing body of evidence had implicated synovial inflammation in the pathogenesis of PTOA. After an injury there is significant hemarthrosis that contributes to cartilage catabolism and decreased proteoglycan synthesis (381).

Pro-inflammatory interleukins (IL-1, IL-6 and IL-10) are associated with a risk of developing OA. Polymorphisms of IL-1 genes and its associated receptors have been linked to knee OA (174). IL-1 stimulates synoviocytes and chondrocytes to secrete inflammatory mediators and proteases (382). IL-1 levels are higher after and ACL injury, correlating to cartilage damage (383). IL-1 receptor antagonist (IL-1Ra) is a natural inhibitor of IL-1, by binding to the IL-1R1 receptor preventing IL-1 from binding without activating the receptor and starting the inflammatory cascade. IL-1Ra has been used clinically in rheumatoid arthritis (RA) with some success, and it supressed endogenous IL-1 and decreased joint swelling in a rat model of RA (274) and a mouse model of RA (384, 385). In spite of IL-1Ra's success in RA, it has not been as clear of IL-1Ra's role in the management of OA.

Several pre-clinical animal models of OA have shown therapeutic benefits of IL-1Ra. IL-1Ra protected against OA in dogs after ACL transection (280, 281), improved pain and preserved articular cartilage and synovium in horses (284), prevented OA progression (282, 386) and decrease inflammation (273) in rabbits. Two clinical trials have also shown positive

outcomes of IL-1Ra injections for short term benefit of established OA (285, 286), and most recently a clinical trial of IL-1Ra injection after an ACL injury showed both a reduction in pain, as well as an increase in function (287).

I therefore aimed to test IL-1Ra therapy to prevent the development of PTOA in a surgically induced inflammatory model of PTOA. I hypothesized that daily intra-articular injections of IL-1Ra Anakinra in rabbit knees for either 72 hours or 3 weeks would decrease both immediate and acute joint inflammation and hence be chondroprotective.

3. MATERIALS AND METHODS

3.1. Surgical methods and animal groups

Thirty-five 1 year old female New Zealand White rabbits were used. Twenty-four animals received experimental bone-injury surgery (called IL-1Ra): 72 hour surgical control (n=6) called SxCtl; 72 hour IL-1Ra (n=6); 3 week surgical control (n=6) SxCtl; and 3 week IL-1Ra (n=6). Eleven normal animals served as time matched non-operated controls (n=5-6/group) to account for inter-animal variability (called Ctl).

Twenty-four rabbits underwent surgery of their right knee to induce bone injury (349); approved by the U of C Animal Care Committee (M08085). A lateral arthrotomy between the extensor tendon and patellar tendon was performed. Deflecting the fat pad, using a drill guide, two 1.1mm drill holes (ECT® internal fracture fixation drill, Zimmer, Warsaw, IN) were drilled to the chondral margins, to a depth of 10mm. The fat pad was then reduced and the incisions closed. Left legs in all cases remained as unoperated contralateral controls. Animals were allowed immediate cage activity after surgery until they were euthanized in groups of 5 or 6 rabbits at 72 hours (72h-Ctl, 72h-SxCtl and 72h-IL-1Ra) or at 3 weeks (3w-Ctl, 3w-SxCtl and 3w-IL-1Ra).

3.2. IL-1Ra treatment

Twelve of the rabbits that received surgery recieved intra-articular injections (similar to clinical applications) with 0.5mg/kg/day IL-1Ra Anakinra (Kineret, Swedish Orphan Biovitrum, Stockholm, SWE). Dosages were based on previous animal studies and clinical dosages for patients. They received their first injection immediately after surgery and then an injection daily until they were euthanized at either 72h (n=6) or 3w (n=6).

At the time of euthanasia, all joint tissues collected from both right and left knees. The joint gross morphology was assessed qualitatively for osteophytes, cartilage lesions, cartilage

discolouration and cartilage fibrillation by two experienced observers. Synovium was sampled from the suprapatellar area and the femoral groove. The fat pad was collected. Cartilage samples were taken from six different areas: patella, femoral groove, lateral and medial femoral condyle, and lateral and medial tibial plateau.

3.3. Histology

Cartilage was embedded in Tissue-Tek® O.C.T.™ (Sakura Finetek USA, Inc., Torrance, CA) and cryosectioned (5μm). Slides were stained with Safranin-O/Fast-green, and graded blind by one observer on two occasions with a modified Mankin score (315) (in press). A minimum of six sections from each area of each joint were analyzed and scores were summed across the areas.

3.4. Real-time reverse transcriptase polymerase chain reaction (qPCR)

The synovium was examined for changes in mRNA expression of: IL-1β, IL-1Ra, IL-6, IL-8 (interleukins), tumor necrosis factor α (TNFα), matrix metalloproteinase 13 (MMP13), transforming growth factor β (TGFβ), basic fibroblast growth factor (bFGF), and monocyte chemoattractant protein 1 (MCP1) (see Appendix 3 Table 2 for primers) using qPCR as described by Reno *et. al.*(317). Samples were run in duplicate. RNA was extracted using a Qiagen RNeasy kit (Qiagen Sciences, Germantown, MD), treated and quantified with DNAse-I. 1μg of total RNA was transcribed to a single stranded cDNA with Qiagen Omniscript RT kit (Qiagen Sciences, Germantown, MD). Primers were made and validated for rabbit TGFβ and MMP13 (Appendix 3 Table 2). iCycler Thermal Cycler (BIO-RAD, Hercules, CA) was used for amplification and detection after SYBR green supermix (BIO-RAD, Hercules, CA), molecular water, and forward and reverse primers were mixed with the samples. Gene expression was normalized to 18S mRNA and iCycler IQ Optical System Software version 3.0a (BIO-RAD, Hercules, CA) was used to quantify the results.

The fat pad and cartilage from the tibial plateaus (pooled) and femoral condyles (pooled) were examined for expression of TGFβ, bFGF, MMP13, IL-8, IL-1Ra, IL-1β, TNFα, and MCP1 with the methods described above.

3.5. Western Blots

The right fat pad and right synovium of non-operated controls, surgical controls and IL-1Ra animals were examined for protein levels.

3.4.1. Protein extraction

Protein was isolated from the Trizol layer following the manufacturer's directions (Qiagen Sciences, Germantown, Maryland) during RNA extraction, described above. The pellets were re-suspended in Tissue Protein Extraction Reagent (T-PER; Pierce, Rockford, Illinois) and EDTA-free halt protease inhibitor cocktail, (Pierce, Rockford, Illinois).

3.4.2. SDS-PAGE and Western blot

Loading samples were prepared and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using NuPAGE 4-12% Bis-Tris gel in 1X MES SDS running buffer at 110V for one and a half hours. Proteins separated on the gel were then transferred to a polyvinylidene fluoride (PVDF) membrane at 20V for one and a half hours. Membranes were incubated with antibodies directed against bFGF (1:750; C-18 sc1360 Santa Cruz Biotecnologies Inc, Santa Cruz, California) or IL-1Ra (1:750; Q-19 sc8481 Santa Cruz Biotecnologies Inc, Santa Cruz, California) or IL-1β (1:1000; R-20 sc1252 Santa Cruz Biotecnologies Inc, Santa Cruz, California) or IL-6 (1:750; M-19 sc1265 Santa Cruz Biotecnologies Inc, Santa Cruz, California) or IL-8 (1:1000; 8M6 ab34100 Abcam, Cambridge, United Kingdom) or TGFβ (1:1000; TB21 ab26969 Abcam, Cambridge, United Kingdom) at 4^0C overnight. Membranes were incubated for one hour at room temperature in an HRP-conjugated secondary antibody: bFGF, IL-1Ra, IL-1β and IL-6 were incubated with anti-goat

IgG (1:5000; NBP1-74800 Novus Biotechnology, Oakville, Ontario), GAPDH, TGFβ and IL-8 were incubated with anti-mouse IgG (1:5000; NBP1-75130 Novus Biotechnology, Oakville, Ontario). The membranes were developed using Novex chemiluminescent substrates (Invitrogen, Burlington, Ontario) and electro-chemiluminescent detection kit (Amersham Biosciences; Buckinghamshire, United Kingdom) and were quantified by densitometry. Antibodies directed against GAPDH (1:2000; GA1R ab125247 Abcam, Cambridge, United Kingdom) were used as a loading control.

3.6. Statistical analysis

Statistical analysis of cartilage Mankin scores was performed with Kruskal-Wallis and Friedman with Tukey's HSD, and ANCOVA with Bonferroni post-hoc (CI=95%). Scores were also compared between joint locations and subscores with ANCOVA with Bonferroni post-hoc (CI-95%). All PCR and western blot data were analyzed using an UNIANOVA with Bonferroni post-hoc and confirmed with a Kruskal-Wallis test with Tukey's HSD post-hoc. All tests used unequal variance assumptions and non-parametric statistics to account for group size differences and variability. $P < 0.05$ was considered significant (SPSS; IBM, Armonk, NY).

4. RESULTS

During the course of the study all animals appeared healthy, they were active in their cages and all ate and drank normally. Surgical animals regained baseline activity moving around their cages within 6 hours after surgery (not quantified).

4.1. Gross and histological data

Synovial fluid was bloody at 72h-SxCtl, but there were minimal signs of cartilage damage. IL-1Ra treated right joints were bloody along the incision at 72h and 3w. 72h-IL-1Ra joints had minimal signs of gross cartilage damage; two animals had grade 1 fissures in the contralateral femoral groove, and two animals had grade 1-2 surface irregularities of the right medial femoral condyle. Signs of gross surface damage were apparent by 3w and cartilage showed some fibrillation (grade 2-3) and discolouration in all 3w-SxCtl animals. 3w-IL-1Ra joints also had signs of some gross cartilage damage in five animals; three animals had small osteophytes (femoral groove, patella, and tibia) of right and left knees, all five had low grade fibrillation and discolouration of the medial tibial plateau of right and left knees, and two animals had low grade fragmentation of the right medial tibial cartilage. (Figure 1a).

These gross findings of cartilage damage were partially supported by blinded histology. There were no differences between subscores; therefore the subscores were pooled for each animal. The medial tibial plateau Mankin cartilage scores were significantly higher than Mankin scores of the femoral groove ($p = 0.007$; MD =1.32; 95%CI =0.22-2.43), lateral tibial plateau ($p = 0.001$; MD =1.56; 95%CI =0.46-2.66), medial femoral condyle ($p < 0.001$; MD =1.79; 95%CI =0.68-2.89), and lateral femoral condyle ($p = 0.006$; MD =1.34; 95%CI =0.23-2.44) of surgical controls corroborating the gross observations (Figure 1b). Patellar scores were also significantly higher that medial femoral condyle scores ($p = 0.007$; MD =1.36; 95%CI =0.22-2.50). There was no difference between the time matched surgical control groups and therefore were pooled

together. Summed surgical control right limb scores were significantly higher than their contralateral limb ($p<0.001$; MD =19.08; 95%CI =6.26-31.90), non-operated controls ($p<0.001$; MD =29.20; 95%CI =16.85-41.56), 72h-IL-1Ra right limb ($p<0.001$; MD =30.23; 95%CI =17.41-43.05), 72h-IL-1Ra left limb ($p<0.001$; MD =31.40; 95%CI =18.58-44.23), 3w-IL-1Ra right limb ($p<0.001$; MD =28.37; 95%CI =15.54-41.19), and 3w-IL-1Ra left limb ($p<0.001$; MD =28.81; 95%CI =15.99-41.63) (Figure 1c). There were no differences between limbs of IL-1Ra treated animals ($p>0.05$) and there were no differences between 72h-IL-1Ra and 3w-IL-1Ra with treatment ($p>0.05$).

4.2. qPCR

4.2.1. Synovium

Interestingly, synovial mRNA expression of TGFβ was significantly higher in right 72h-IL-1Ra joints than non-operated controls ($p = 0.005$; MD = 0.63; 95%CI = 0.12 to 1.15), 72h-SxCtl ($p = 0.005$; MD = 0.77; 95%CI = 0.14 to 1.40), 3w-SxCtl ($p=0.001$; MD = 0.80; 95%CI = 0.22 to 1.38), 3w-IL-1Ra ($p <0.001$; MD = 0.78; 95%CI = 0.26 to 1.30) (Figure 2a). There were no significant differences in IL-1Ra, IL-1β, IL-6, IL-8, TNFα. MMP13 and MCP1 ($p>0.05$).

4.2.2. Fat pad

Fat pad expression of IL-1Ra was 4-5 fold higher in72h-IL-1Ra than time non-operated controls ($p <0.001$; MD = 2.51; 95%CI = 1.01 to 4.06), 72h-SxCtl ($p = .014$; MD = 1.71; 95%CI = 0.21 to 3.21), and 3w-SxCtl ($p <0.01$; MD = 2.48; 95%CI = 0.98 to 3.98) (Figure 2b). There was a 4-5 fold increase in TGFβ expression after 72h of IL-1Ra treatment and a 4-8 fold increase in TGFβ expression after 3w of IL-1Ra treatment compared to time matched non-operated controls ($p =0.013$; MD = 3.76; 95%CI = 0.47 to 7.45 and $p <0.001$; MD = 6.37; 95%CI = 3.08 to 9.66 respectively), 72h-SxCtl ($p =0.019$; MD = 3.64; 95%CI = 0.35 to 6.93 and $p <0.001$; MD = 6.25; 95%CI = 2.96 to 9.54 respectively), and 3w-SxCtl ($p =0. 026$; MD = 3.54; 95%CI = 0.25

to 6.83 and p <0.001; MD = 6.15; 95%CI = 2.86 to 9.44 respectively) (Figure 2c). Anakinra increased bFGF expression 5-7 fold in both 72h-IL-1Ra and 3w-IL-1Ra than time matched non-operated controls (p =0.001; MD = 2.83; 95%CI = 0.76 to 4.89 and p <0.001; MD = 4.36; 95%CI = 2.30 to 6.42 respectively), 72h-SxCtl (p <0.001; MD = 3.17; 95%CI = 1.11 to 5.24 and p <0.001; MD = 4.71; 95%CI = 2.65 to 6.77 respectively), and 3w-SxCtl (p =0. 009; MD = 2.45; 95%CI = 0.39 to 4.51 and p <0.001; MD = 3.98; 95%CI = 1.92 to 6.04 respectively) (Figure 2d). There was significantly higher (7 fold) TNFα expression in 3w-IL-1Ra than 3w non-operated control (p = 0.012; MD = 4.54; 95%CI = 0.62 to 8.47), 72h-SxCtl (p = 0.027; MD = 4.19; 95%CI = 0.27 to 8.11), and 3w-SxCtl (p = 0.025; MD = 4.22; 95%CI = 0.30 to 8.14) (Figure 2e). There was also an increase in the ratio of IL-1β:IL-1Ra both 72h-IL-1Ra and 3w-IL-1Ra compared with surgical controls. There were no differences in the expression of IL-1β, MCP1, IL-8, or MMP13 in the fat pad between the groups.

4.2.3. Cartilage

The cartilage, tibial plateaus pooled medial and lateral and femoral condyles pooled medial and lateral, had no significant difference between groups in IL-1Ra expression, MCP1 expression, IL-6 expression, IL-8 expression, and IL-1β expression. There were also no significant differences in bFGF and TNFα expression in the tibial plateaus pooled medial and lateral and no significant differences in MMP13 expression in the femoral condyles pooled medial and lateral. TGFβ expression in the tibial plateaus was 4 fold higher in 3w-SxCtl than 3w-IL-1Ra (p = 0.041; MD = 2.45; 95%CI = 0.06 to 4.84) (Figure 3a). 3w-SxCtl also had significantly higher MMP13 expression (5 fold) than 72h-IL-1Ra (p = 0.042; MD = 3.81, 95%CI = 0.08 to 7.54) and 3w-IL-1Ra (p = 0.039; MD = 3.85; 95%CI = 0.12 to 7.57) (Figure 3b). There was significantly higher TNFα, bFGF, and TGFβ (Figure 3c-e) expression in the femoral condyles of 72h-SxCtl. TNFα was 7-8 fold higher than 3w-SxCtl (p = 0.022; MD = 0.63; 95%CI

= 0.06 to 1.20), 72h-IL-1Ra (p = 0.036; MD = 0.52; 95%CI = 0.02 to 1.01), and 3w-IL-1Ra (p = 0.007; MD = 0.62; 95%CI = 0.13 to 1.11). bFGF was 4 fold higher than non-operated controls (p = 0.013; MD = 3.89; 95%CI = 0.45 to 7.33), 3w-IL-1Ra (p = 0.012; MD = 3.91; 95%CI = 0.48 to 6.86), and 72h-SxCtl contralateral limb (p = 0.025; MD = 4.25; 95%CI = 0.28 to 8.22). TGFβ was 8 fold higher than non-operated controls (p = 0.019; MD = 7.30; 95%CI = 0.64 to 13.96), 3w-IL-1Ra (p = 0.047; MD = 7.74; 95%CI = 0.05 to 15.44), and 72h-SxCtl contralateral limb (p = 0.022; MD = 7.21; 95%CI = 0.55 to 13.87). In summary, TGFβ and MMP13 were significantly higher in the tibial plateaus of 3w-SxCtl compared to IL-1Ra animals. TNFα, bFGF, and TGFβ were significantly higher in the femoral condyles of 3w-SxCtl compared to IL-1Ra animals.

4.3. Western Blots

In the synovium of injured joints there were significantly lower levels of IL-1β protein after treatment with Anakinra compared to SxCtl for all time points (p = 0.036; MD = 0.32; 95%CI = 0.19 to 0.69) (Figure 4a). There was a trend for lower protein levels of IL-6 in the synovium in 72h-IL-1Ra and 3w-IL-1Ra than 7h2-SxCtl and 3w-SxCtl (p>0.05) (Figure 4b).

Protein levels of IL-1Ra were significantly lower after treatment with Anakinra compared to SxCtl for all time points in the fat pad (p = 0.015; MD = 0.39; 95%CI = 0.08 to 0.87) (Figure 4c). IL-6 levels in the fat pad were significantly higher in 72h-IL-1Ra than 72h-SxCtl (p = 0.044; MD = 0.06; 95%CI = 0.05 to 0.71) (Figure 4d). While not significant, there was a trend for lower IL-8 after treatment with Anakinra (p>0.05), and there was a decrease in the ratio of IL-1β/IL-1Ra (Figure 5e-f).

5. FIGURES

5.1. Figure 1 Cartilage histology

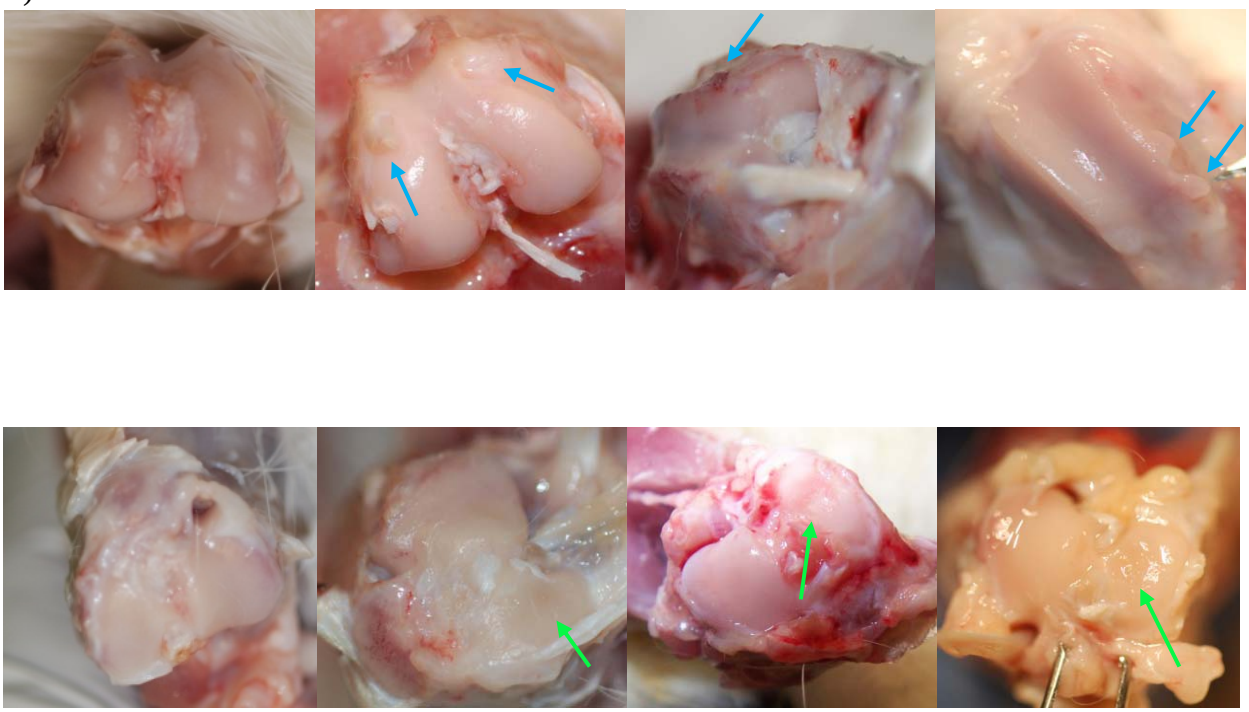
Figure 1. a) Articular cartilage wear of the femur/femoral groove (top row) and tibia (bottom row). From left to right: non-operated Ctl, 3w-SxCtl, 72h-IL-1Ra, and 3w-IL-1Ra. Note 72h-SxCtl is not depicted as it was identical in appearance to non-operated Ctl. By 3 weeks post-surgery the cartilage has some fibrillation of the medial tibial plateau and osteophytes on the femur. 72h-IL-1Ra also had fibrillation of the tibia plus discoloration and osteophytes on the femoral groove. 3w-IL-1Ra had slightly more marked tibial plateau defects and larger more prominent osteophytes than 72h-IL-1Ra. Green arrows denote fibrillation/focal cartilage defect; blue arrows denote osteophytes.

b) MTP (medial tibial plateau) had significantly higher scores ($p>0.05$) than the other cartilage locations.

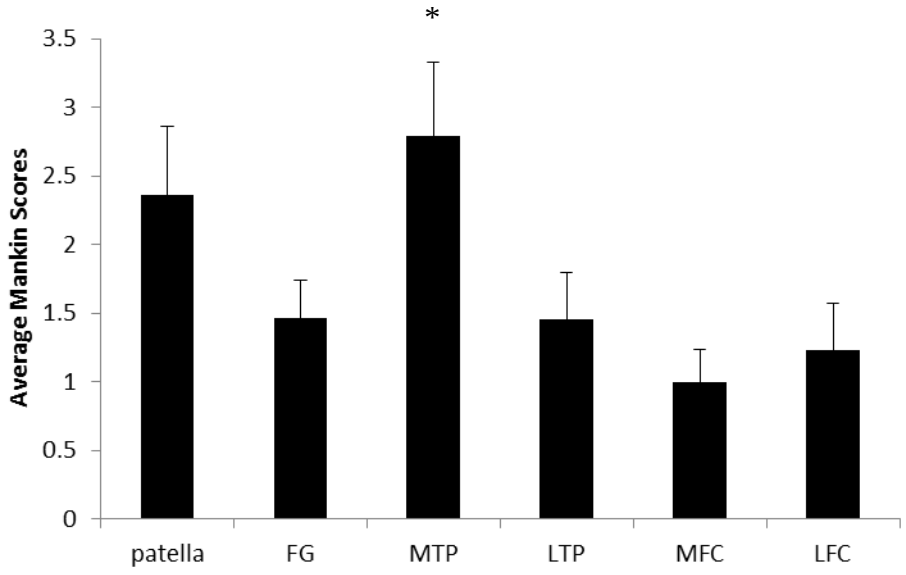
c) 72h/3w-SxCtl pooled had significantly higher Mankin scores ($p<0.05$) that other groups.

* = significant difference ($p<0.05$)

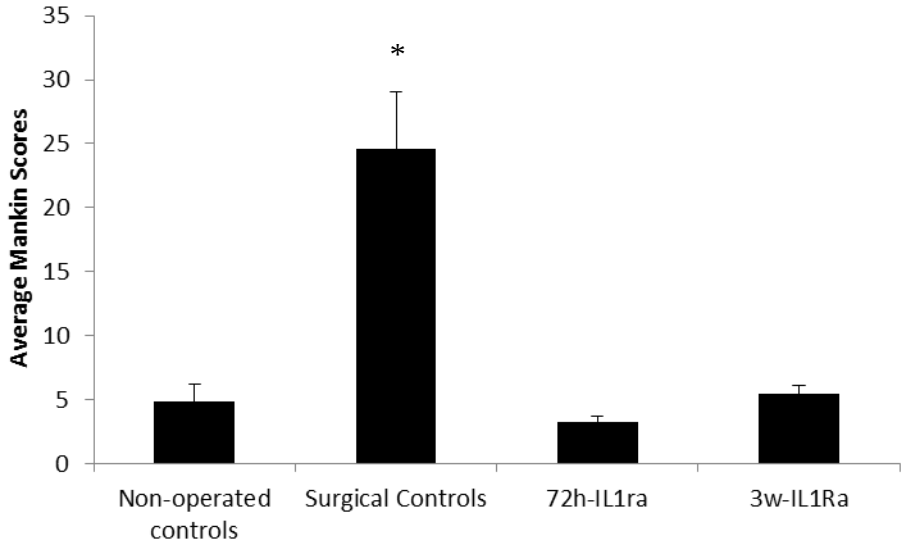
a)



b)


*
3.5
3
2.5
2
1.5
1
0.5
0
Average Mankin Scores
patella
FG
MTP
LTP
MFC
LFC

c)


*
35
30
25
20
15
10
5
0
Average Mankin Scores
Non-operated controls
Surgical Controls
72h-IL1ra
3w-IL1Ra

5.2. Figure 2 Synovial and Fat pad qPCR

Figure 2. a) A dot blot of synovial tissue demonstrating the range and variability in the animal data. TGFβ was significantly higher in 72h-IL-1Ra.

b) A dot blot of fat pad demonstrating the range and variability in the animal data.IL-1Ra in 72h-IL-1Ra fat pad was significantly higher.

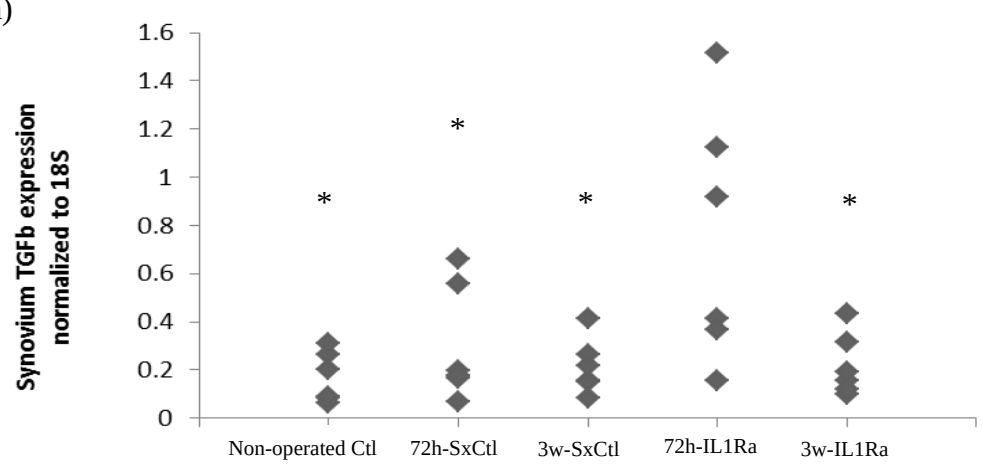
c) A dot blot of fat pad demonstrating the range and variability in the animal data.TGFβ was significantly higher in 72h-IL-1Ra and 3w-IL-1Ra fat pad.

d) A dot blot of fat pad demonstrating the range and variability in the animal data.bFGF was significantly higher in 72h-IL-1Ra and 3w-IL-1Ra fat pad.

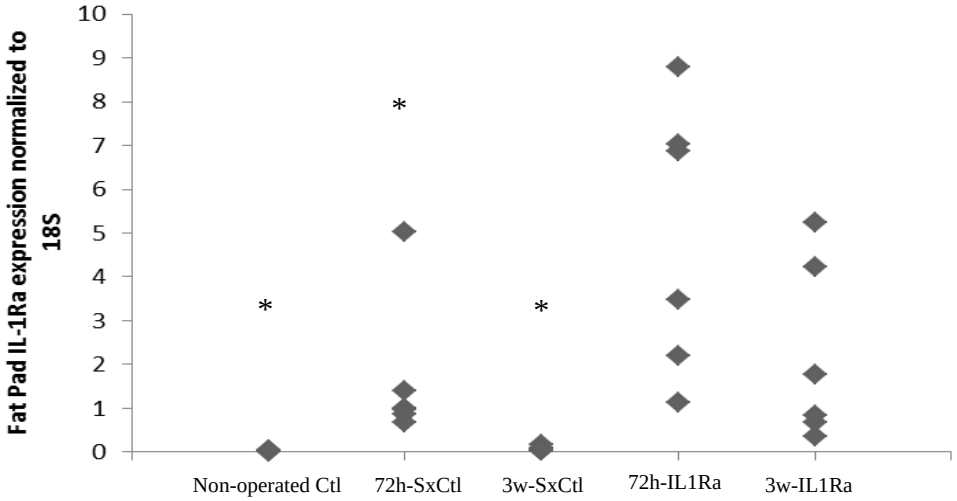
e) A dot blot of fat pad demonstrating the range and variability in the animal data.TNFα was significantly higher in 3w-IL-1Ra fat pad.

n=6/group.* = significant difference ($p<0.05$) than 72h-IL-1Ra, δ = significant difference ($p<0.05$) than 3w-IL-1Ra.

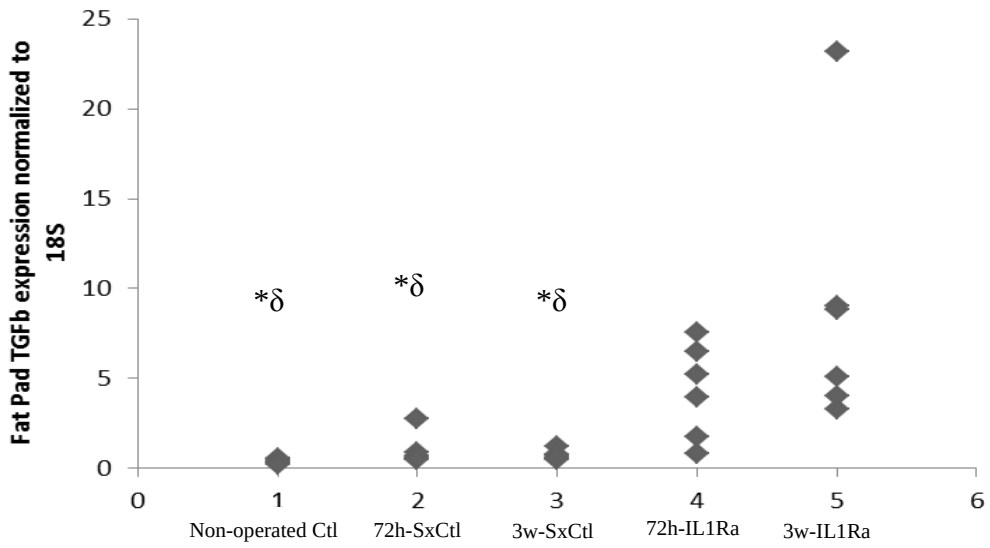
a)


Synovium TGFb expression normalized to 18S
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
*
Non-operated Ctl
72h-SxCtl
3w-SxCtl
72h-IL1Ra
3w-IL1Ra

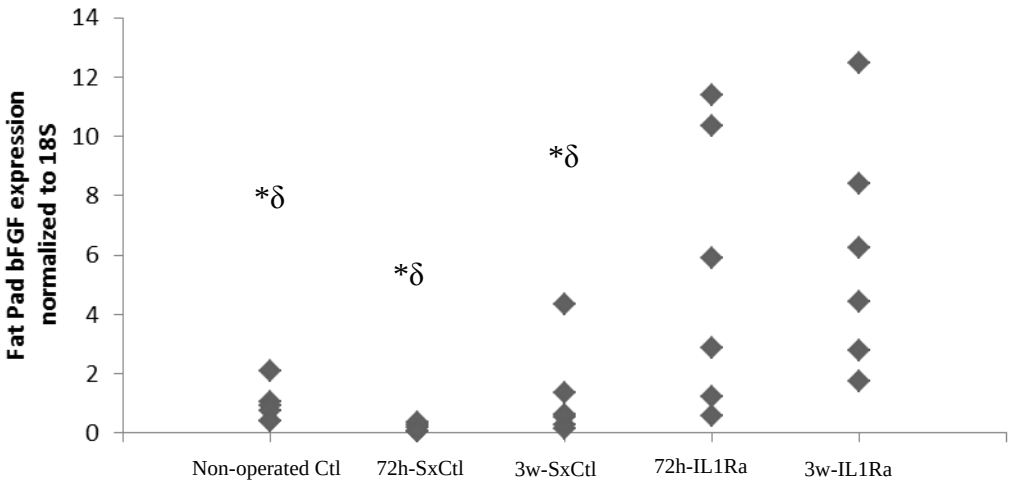
b)


Fat Pad IL-1Ra expression normalized to 18S
10
9
8
7
6
5
4
3
2
1
0
*
*
*
Non-operated Ctl
72h-SxCtl
3w-SxCtl
72h-IL1Ra
3w-IL1Ra

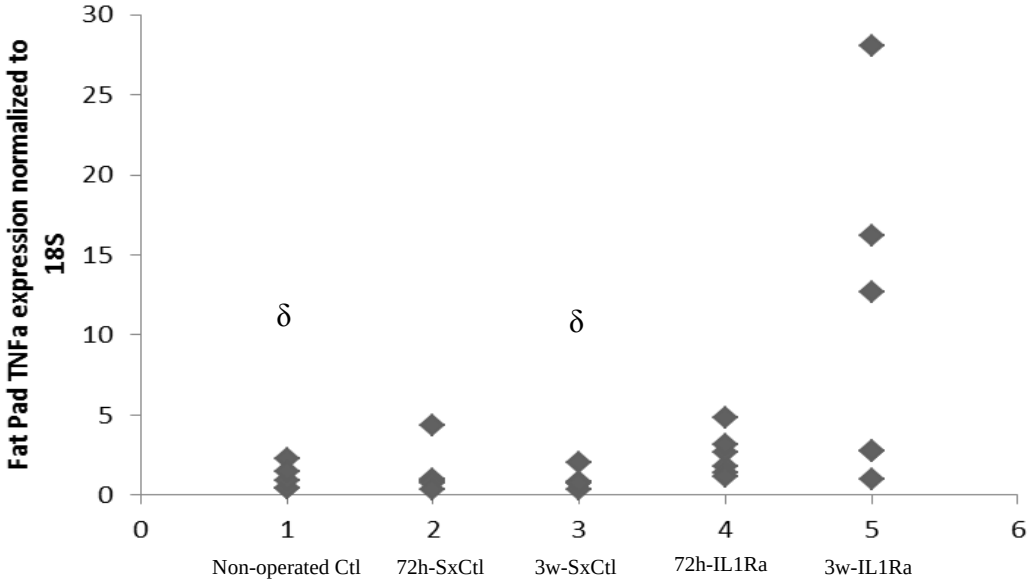
c)


Fat Pad TGFb expression normalized to 18S
25
20
15
10
5
0
*δ
*δ
*δ
0
1
2
3
4
5
6
Non-operated Ctl
72h-SxCtl
3w-SxCtl
72h-IL1Ra
3w-IL1Ra

d)


Fat Pad bFGF expression normalized to 18S
14
12
10
8
6
4
2
0
*δ
*δ
*δ
Non-operated Ctl
72h-SxCtl
3w-SxCtl
72h-IL1Ra
3w-IL1Ra

e)


Fat Pad TNFa expression normalized to 18S
30
25
20
15
10
5
0
δ
δ
0
1
2
3
4
5
6
Non-operated Ctl
72h-SxCtl
3w-SxCtl
72h-IL1Ra
3w-IL1Ra

5.3. Figure 3 Cartilage qPCR

Figure 3. a) A dot blot showing the range and variability in between animals. 3w-SxCtl had significantly higher TGFβ in the tibial plateau.

b) A dot blot showing the range and variability in between animals. 3w-SxCtl had significantly higher MMP13 in the tibial plateau.

c) A dot blot showing the range and variability in between animals. 72h-SxCtl femoral condyle had significantly higher TNFα expression.

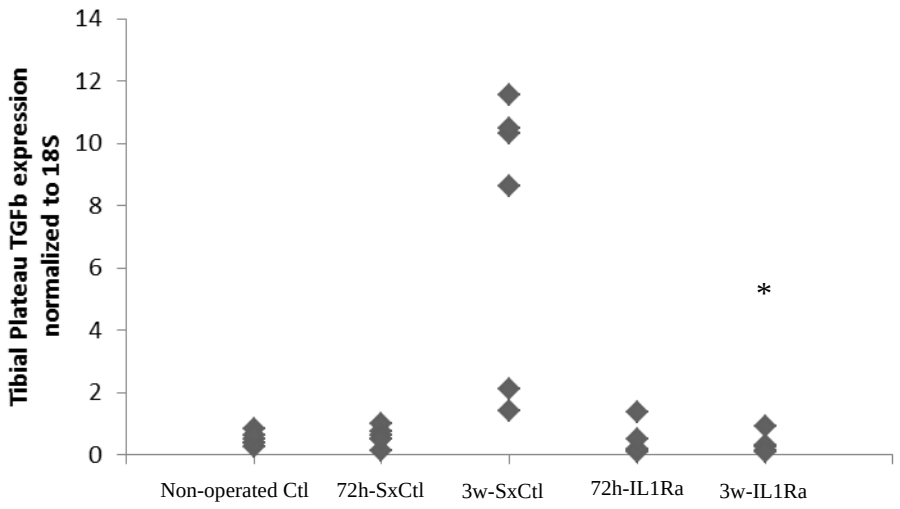
d) A bar graph of right and left hind limbs. 72h-SxCtl femoral condyle had significantly higher bFGF expression.

e) A bar graph of right and left hind limbs. 72h-SxCtl femoral condyle had significantly higher TGFβ expression.

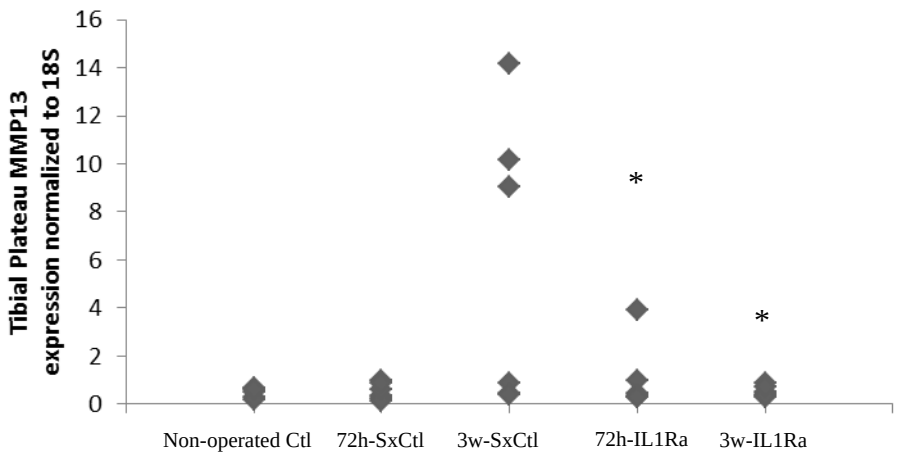
Hatched bars are right limb and solid bars are left limb.

n=6/group. Means and SD are represented in the graphs. * =significantly different ($p<0.05$) than 3w-SxCtl; δ =significantly different ($p<0.05$) than 72h-SxCtl.

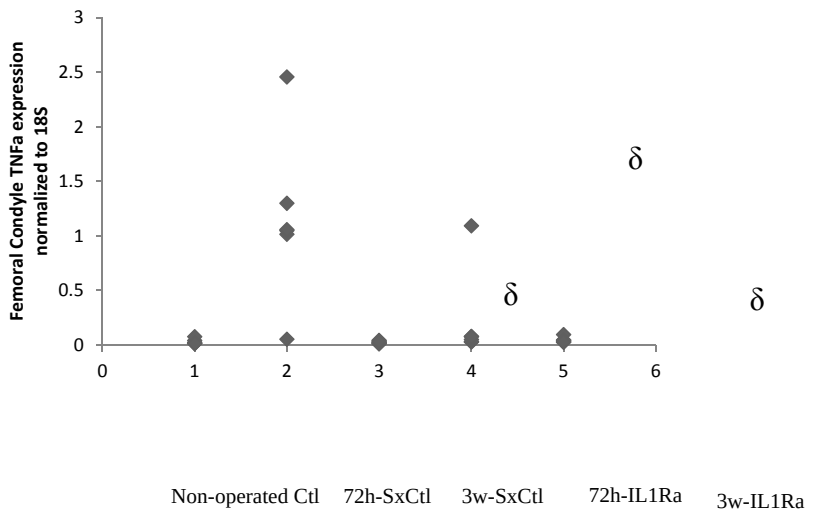
a)


Tibial Plateau TGFb expression normalized to 18S
14
12
10
8
6
4
2
0
*
Non-operated Ctl
72h-SxCtl
3w-SxCtl
72h-IL1Ra
3w-IL1Ra

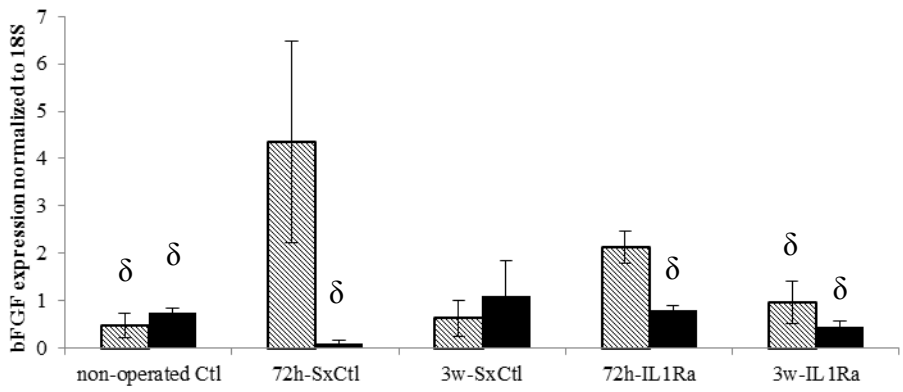
b)


Tibial Plateau MMP13 expression normalized to 18S
16
14
12
10
8
6
4
2
0
*
*
Non-operated Ctl
72h-SxCtl
3w-SxCtl
72h-IL1Ra
3w-IL1Ra

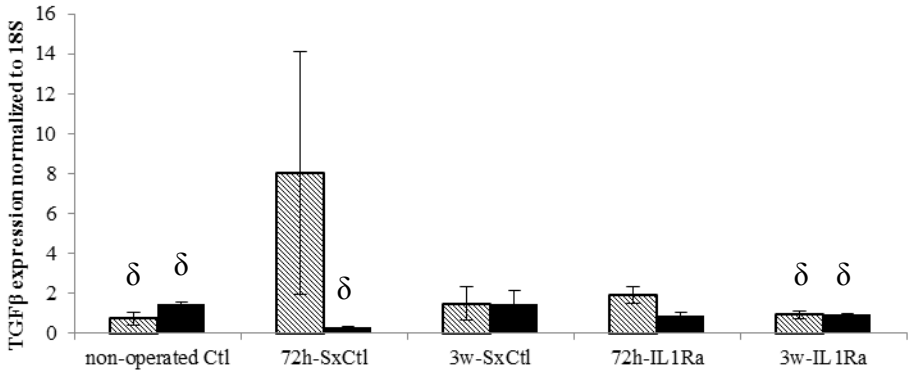
c)


Femoral Condyle TNFa expression normalized to 18S
3
2.5
2
1.5
1
0.5
0
δ
δ
δ
0
1
2
3
4
5
6
Non-operated Ctl
72h-SxCtl
3w-SxCtl
72h-IL1Ra
3w-IL1Ra

d)


bFGF expression normalized to 18S
7
6
5
4
3
2
1
0
δ
δ
δ
δ
δ
δ
δ
non-operated Ctl
72h-SxCtl
3w-SxCtl
72h-IL1Ra
3w-IL1Ra

e)


TGFβ expression normalized to 18S
16
14
12
10
8
6
4
2
0
δ
δ
δ
δ
δ
non-operated Ctl
72h-SxCtl
3w-SxCtl
72h-IL1Ra
3w-IL1Ra

5.4. Figure 4 Synovial and fat pad western blot protein levels

Figure 4. a) Synovial IL-1β was significantly lower in 72h-IL-1Ra and 3w-IL-1Ra.

b) Synovial IL-6 was lower with Anakinra treatment.

c) Fat pad IL-1Ra was significantly higher in 72h-IL-1Ra and 3w-IL-1Ra.

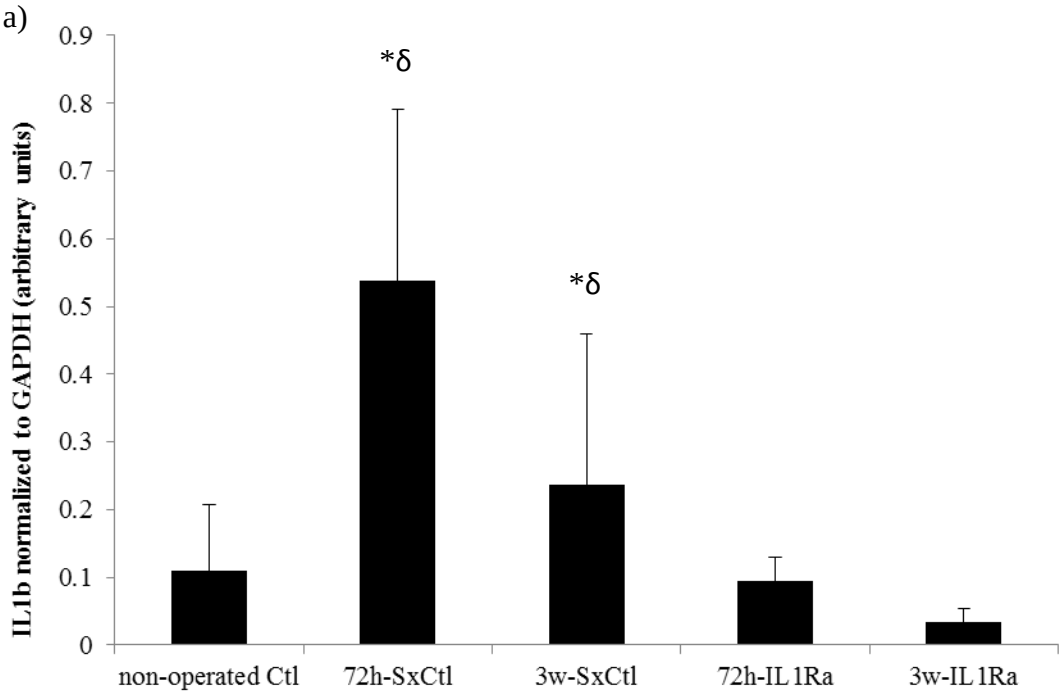
d) Fat pad IL-6 was significantly higher in 72h-IL-1Ra.

e) Fat pad IL-8 was lower with Anakinra treatment.

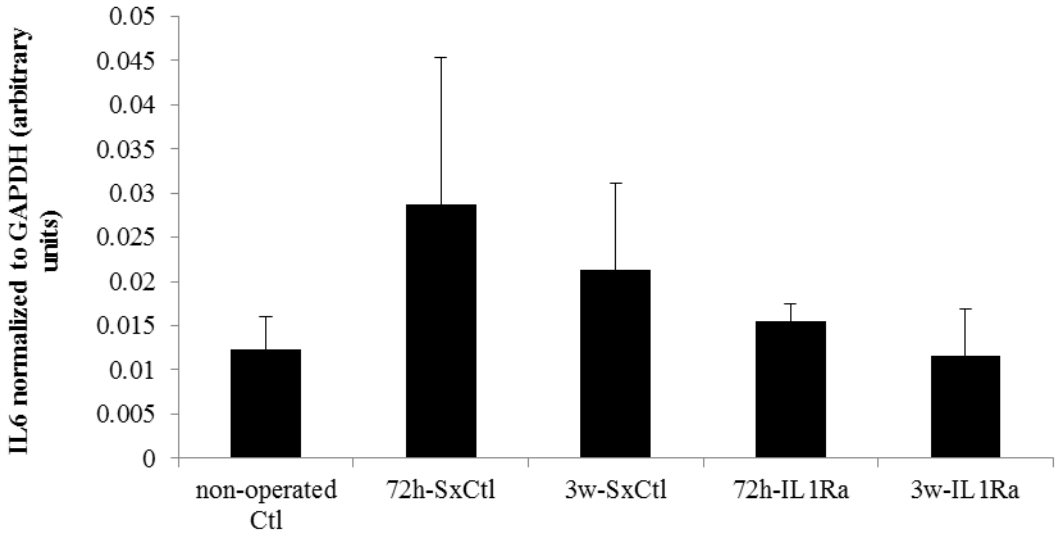
f) The ratio of IL-1β/IL-1Ra in the fat pad was lower in 72h-IL-1Ra and 3w-IL-1Ra.

n=4/group. Means and SD are represented in the graphs. * = significant difference ($p<0.05$) than 72h-IL-1Ra, δ = significant difference ($p<0.05$) than 3w-IL-1Ra.

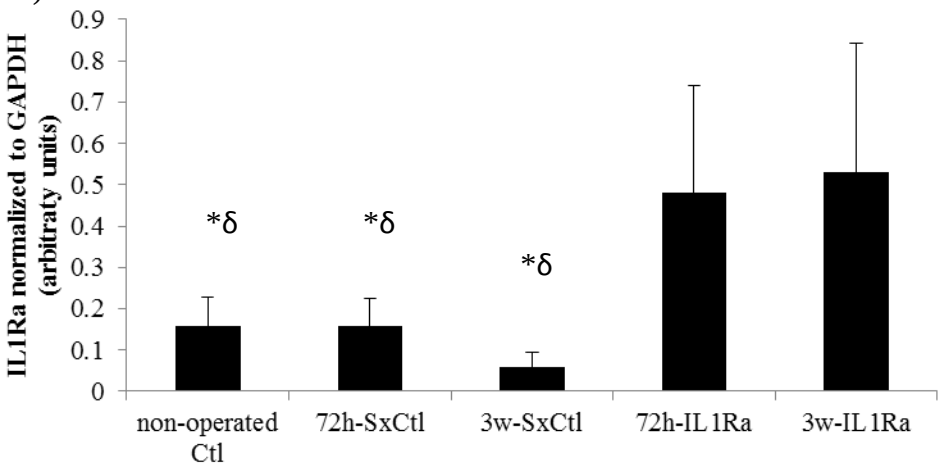
a)


IL1b normalized to GAPDH (arbitrary units)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*δ
*δ
non-operated Ctl
72h-SxCtl
3w-SxCtl
72h-IL 1Ra
3w-IL 1Ra

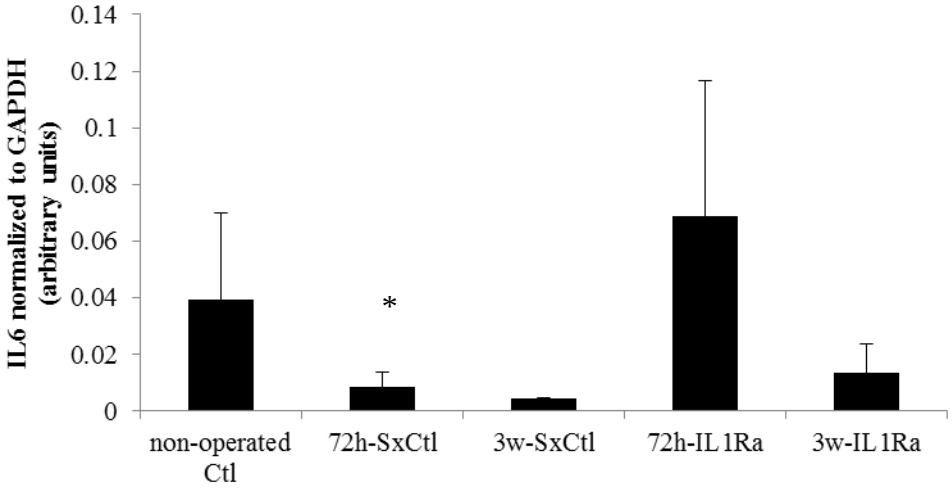
b)


IL6 normalized to GAPDH (arbitrary units)
0.05
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
non-operated Ctl
72h-SxCtl
3w-SxCtl
72h-IL 1Ra
3w-IL 1Ra

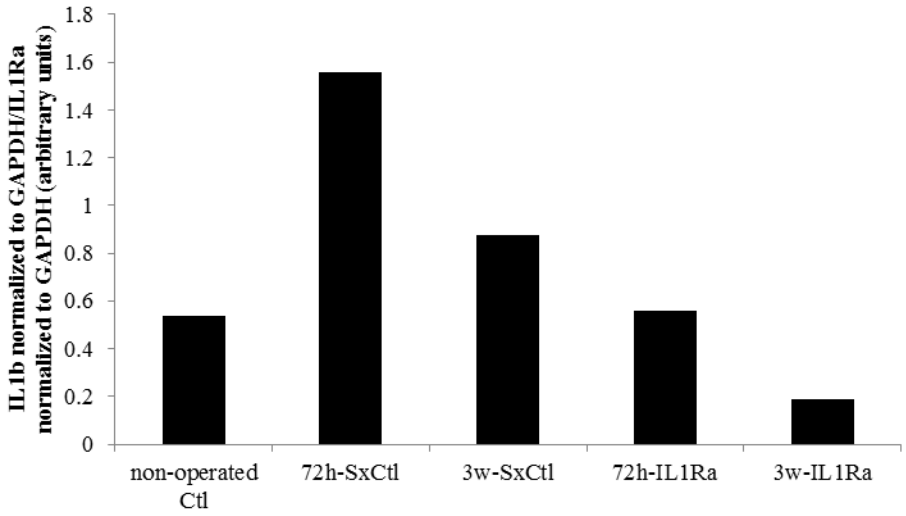
c)


IL1Ra normalized to GAPDH (arbitraty units)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*δ
*δ
*δ
non-operated Ctl
72h-SxCtl
3w-SxCtl
72h-IL1Ra
3w-IL1Ra

d)


IL6 normalized to GAPDH (arbitrary units)
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
*
non-operated Ctl
72h-SxCtl
3w-SxCtl
72h-IL1Ra
3w-IL1Ra

e)


IL1b normalized to GAPDH/IL1Ra normalized to GAPDH (arbitrary units)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
non-operated Ctl
72h-SxCtl
3w-SxCtl
72h-IL1Ra
3w-IL1Ra

6. DISCUSSION AND CONCLUSIONS

The inflammatory cytokine IL-1β has been implicated in the pathogenesis of OA and PTOA. After an ACL injury IL-1 levels are elevated, and that increase has been correlated to cartilage damage in humans and various animal models (383). IL-1 stimulates the production and secretion of inflammatory mediators and proteases by synoviocytes and chondrocytes (382). IL-1 receptor antagonist (IL-1Ra) is a natural inhibitor of IL-1 preventing the inflammatory cascade. It has been used for the treatment of RA in patients' refractory to other treatment modalities. However, much less work has been done relating to its use in OA. I sought to inhibit inflammation post-injury in my new model of PTOA and to prevent cartilage damage using intraarticular injection of IL-1Ra. I specifically hypothesized that daily IL-1Ra treatments given intraarticularly would decrease joint inflammation and prevent PTOA in my rabbit model.

This study showed that high dose Anakinra given repeatedly in this way was chondroprotective at a histological level, similar to previous studies in rabbits, rats and mice demonstrating improved histological scores after treatment with IL-1Ra (280-282, 284). Based on these studies human IL-1Ra appears to have an effect in rabbits. While I found that Anakinra was slightly protective at the histological level, at the gross level there were signs of bone changes and some cartilage damage 3 weeks after surgery regardless of treatment. Histological scores have been correlated to gross scores in animals (353) and in arthroscopic evaluation of the joint (387). It seems likely that the areas of gross damage were not all sampled as they were not targeted; however this is an unlikely reason to have missed any damage, as large samples of cartilage were taken for histological analysis and therefore would have detected gross damage. I also failed to measure the osteophyte and lesion sizes which may have been smaller in treated animals as previously demonstrated in rabbits by Fernandes *et al.* (282). As histological assessment is the gold standard for animal OA, it is likely that IL-1Ra was chondroprotective in

my model. IL-1Ra has been shown to down-regulate MMPs and promote collagen synthesis and GAGs in cartilage explants (196). I was unable to replicate previous results demonstrating a decrease in cartilage MMP expression after treatment with IL-1Ra (280, 388). As seen previously in my model (349) there was an up-regulation of TGFβ expression in both femoral condyles and tibial plateaus. TGFβ is integral to cartilage maintenance and its down-regulation has been correlated to cartilage damage and OA (329). The up-regulation of TGFβ induces synovial fibrosis and osteophyte formation (330, 331). Perhaps the disruption of bone in my model causes changes in bone morphogenetic protein, part of the TGF family and leading to an up-regulation of TGFβ. IL-1Ra treatment was able to significantly reduce these levels likely protecting the synovium. bFGF was also down-regulated with Anakinra in my model. bFGF has an antagonistic effect on the anabolic activities of cartilage and inhibits the synthesis and release of proteoglycans (389), it also activates MAPK and NFκB in human chondrocytes (390). Therefore it is possible that in my model of PTOA IL-1Ra is partially chondroprotective preventing the inhibition of cartilage anabolism and further joint inflammation through the NFκB pathway. However, it fails to fully prevent gross cartilage damage as it was unable to inhibit MMPs in the cartilage.

Despite partially conflicting results in the ability of IL-1Ra treatments to prevent cartilage damage in my model, it was able to reduce inflammation in both the synovium and fat pad. Interestingly, while cartilage TGFβ was decreased with IL-1Ra there was increased TGFβ expression in both the synovium and fat pad after treatment. TGFβ has a close relationship with IL-1β in the joint, therefore it is possible that inhibition of IL-1β in the synovium and fat pad with Anakinra has led to the up-regulation of TGFβ in order to promote cartilage homeostasis. It is clear that different joint tissues react differently to ILRa which may be why there was only

partial chondroprotection. As seen in the cartilage there was increased expression of bFGF in the fat pad corroborating IL-1Ras effects on preventing joint inflammation.

Anakinra was able to result in a profound increase in IL-1Ra expression and protein levels in the fat pad. The fat pad is metabolically active and has been linked recently with the development of OA (333). Anakinra appears to have an effect on the fat pad by increasing IL-1Ra and decreasing the ratio of IL-1β to IL-1Ra. It is possible that IL-1Ra exerts its effects on the fat pad in my model of PTOA causing down-stream changes in IL-1β in the synovium seen here and in previous work (391) and preventing synovial fibrosis (392). IL-1Ra treatment decreases synovial inflammation and synovial membrane thickening (280, 282), which likely prevents further joint inflammation and prevents catabolism of the cartilage.

Unfortunately, due to small sample sizes and variability in the data several markers of inflammation were not significantly different with treatment, despite trends to that effect. In addition, I did not examine the synovial fluid which would have been beneficial in determining further the different effect IL-1Ra has on various joint tissues. Lastly, Anakinra has a very short half-life therefore it is feasible that even with the daily intraarticular administration it failed to remain in the joint long enough to have its full effect. As such, other vehicles for delivering IL-1Ra such as gene transfection may have a greater anti-inflammatory effect and confer better cartilage protection. In addition daily injections are cumbersome and impractical for patient use.

This study is the first to date to investigate the use of IL-1Ra for the prevention of cartilage damage in a model of PTOA which does not cause gross biomechanical changes post-injury. In this model Anakinra had a profound effect of fat pad inflammation, likely resulting in decreased synovial inflammation and at least some partial cartilage protection via the NFκB pathway. Since IL-1Ra inhibits IL-1β and downstream inflammatory events, this study suggests that the IL-1β pathway is only part of the inflammation story involved in the pathogenesis of

PTOA. Therefore it is possible that using IL-1Ra treatment in combination with other anti-inflammatories targeted at the pathogenesis of PTOA or targeting things more upstream in the inflammatory cascade may be more effective in preventing cartilage damage post-injury in this model.

Chapter 5. TRIPEPTIDE feG DOES NOT PREVENT PTOA IN A RABBIT MODEL

1. ABSTRACT

The pathogenesis of osteoarthritis (OA) is in part due to joint inflammation. Many cells and immunomodulators have been implicated in the development of OA. Chemokines attract leukocytes to a site of injury where they bind to integrins and adhesion molecules for chemotaxis and emigration. The tripeptide feG has been shown to interfere with cell adhesion via a variety of proposed mechanism, with a secondary reduction in oxidative bursts, reactive oxygen species (ROS) and inflammation in some model systems, both in vitro and in vivo. Based on this evidence I hypothesized that feG administered systemically immediately before surgical injury would inhibit the development of post-traumatic OA in my rabbit model. In order to examine this hypothesis in a pilot study, nine rabbits were used: three non-operated normal controls, three surgery + saline injection sham controls, and three experimental surgical animals in which feG was given intravenous (IV) 5 minutes prior to the surgical joint injury. All animals were euthanized 72 hours after surgery at which time tissues were collected and analyzed in a blinded fashion with Mankin score and qPCR. Results showed no significant differences in cartilage histology or mRNA expression of the synovium and fat pad (TNFα, MCP1, IL-6, IL-8, and IL-1β) between experimentals and shams. There are several possibilities as to why feG failed to prevent OA in this model, including: feG inhibits neutrophil adhesion, however, neutrophils are not as important in the development of OA, resident synovial macrophages are key players in joint OA and therefore are not inhibited by feG, the time course or dosage of treatment were insufficient to prevent OA, and lastly IV administration may fail to penetrate the joint. In my rabbit model we nonetheless conclude that feG failed to prevent post-traumatic OA and I therefore did not do a larger study with this proposed inhibitor.

2. INTRODUCTION

The tripeptide feG is a metabolically stable form of FEG (Phe-Glu-Gly) isolated from rat salivary glands known to be immunoregulatory (393) and inflammatory/anti-inflammatory agents (394). Compared with current anti-inflammatories, such as NSAIDs and corticosteroids which work via enzymatic block, feG is considered a new class of anti-inflammatory known as Immune Selective Anti-Inflammatory Derivatives which down-regulate integrins thereby inhibiting leukocyte adhesion, chemotaxis and migration. feG also inhibits the production of reactive oxygen species (ROS) by activated neutrophils. feG has been shown to have a long biological half-life with affects lasting a minimum of 24 hours (395).

feG prevents polymorphonuclear leukocytes (PMNs) and monocyte migration and decreases the amount of intergrin-CD18 expression (395, 396) and regulates the binding of PMNs (395). It also decreases levels of CD49d and α4-integrin, which is produced by neutrophils (397), suggesting feG prevented PMN accumulation. Initial work on feG demonstrated its potential to decrease hypotension in cardiovascular anaphylaxis and inhibit adverse outcomes in intestines (398). feG was also able to reduce PMN infiltration in the spinal cord after injury in rats (399, 400). There was a decrease in myeloperoxidase activity after acute treatments with feG (399) suggestive of decreased PMN infiltration. Delaying treatment with feG after injury was unable to prevent PMN transmigration. The mechanism by which feG has been suggested to work is by inhibiting the adhesion of leukocytes to fibrinogen and fibronectin, vitronectin and heparin.

In other studies, feG has also been shown to regulate the production of reactive oxygen species (ROS) independent of PMN recruitment. Bao *et al.* (399) showed a decrease in myeloperoxidase a ROS released from PMNs and a decrease concentration of free radicals and theorized that this was because of the inhibition of integrins and decrease PMN migration (401).

However, in rats feG was able to decrease intracellular oxidative burst of circulating PMNs by preventing a loss of responsiveness to PKCδ (rottlerin) inhibitors on circulating PMNs (402).

Further work on feG has shown that recruitment of both eosinophils (Eo) and macrophages (Mϕ) was inhibited by feG (403). feG reduced ICAM expression on Eo and Mϕ (403, 404) explaining the reduction in these cells post-treatment (405, 406). Prophylactic treatment with feG also reduced ICAM-1 expression (407).

feG also appears to play a role in decreasing pro-inflammatory cytokines, possible via NFκB activity. When rats were treated with feG after lung inflammation was induced there was a marked reduction in TNFα (403). TNFα, INFγ and IL4, however, do not appear to change with feG treatment of feline asthma (405, 406).

In all of these previous studies leukocyte activation or sensitization was required for feG to inhibit transmigration and superoxide production *in vivo*. However, in a recent study neutrophil priming or prior sensitization was not required to ameliorate disease with both prophylactic and therapeutic feG (408). This is critical for its potential use in non-sensitized inflammatory conditions expanding its therapeutic potential.

3. RATIONALE

Post-traumatic osteoarthritis (PTOA) has a definite inflammatory component critical in its pathogenesis as demonstrated in the previous studies discussed in Chapters (2-4). Therefore if the damaging components of the inflammatory process could be blocked it is possible that early intervention could modify the disease process preventing PTOA. One proposed mechanism to target is that of leukocyte transmigration. It is known that synovial fibroblasts activate endothelial cells and induce leukocyte adhesion in OA (409). In acute synovitis these leukocytes are neutrophils, however in chronic synovitis seen in OA these recruited leukocytes are predominantly Mϕ with some lymphocytic infiltrate (410). While the effect of feG on leukocyte transmigration and chemotaxis has mainly been inhibiting neutrophil adhesion via integrin, some of the work has shown it inhibits Mϕ adhesion via ICAM-1 as well. Therefore, I wanted to examine the role of feG in potentially preventing the development of PTOA.

4. HYPOTHESIS AND AIMS

I hypothesized that treatment with feG after surgery in the rabbit model would prevent leukocyte recruitment post-injury thereby preventing cartilage damage and PTOA. I tested this hypothesis by doing a pilot project injecting three New Zealand White rabbits with a single 500 µl/kg dose of intravenous feG after surgery to induce OA without destabilizing the joint or altering joint loading mechanics.

5. MATERIALS AND METHODS

5.1. Animal Treatments

Nine skeletally mature one year old female New Zealand White (NZW) rabbits were obtained from Reimans Furriers (St. Agatha, ON, Canada). Previous radiographic studies of rabbits from this supplier (411) have confirmed that skeletal maturity occurs at around 10 months of age. The rabbits were housed and treated at the University of Calgary, Faculty of Medicine Animal Vivarium in accordance with the guidelines of the Canadian Council on Animal Care and under the approval of the Faculty of Medicine Animal Care Committee (protocol number M08085). Animals were housed for two weeks after arrival prior to any interventions in order to allow the rabbits adequate time to acclimatize.

Six rabbits underwent an intra-condylar drill surgery (BI) in order to injure the joint by creating an isolated bone injury which would not change the joint mechanics or joint loading in the rabbit knee. The rabbits were treated with Fluoroquinolone Enrofloxacin (Baytril®, Bayer HealthCare Animal Health, Monheim, Germany) two days prior to surgery, the day of surgery and three days post-surgery in order to minimize the risk of post-surgical infection. Immediately prior to surgery (pre-anesthetic) rabbits were given a dose of 0.9mg/kg of Acepromazine Maleate intravenous (IV) and a dose of 0.01mg/kg of buprenorphine hydrochloride (Buprenex®, Reckitt Benckiser Ltd., Slough, United Kingdom) subcutaneous (SC). During surgery rabbits were anesthetised with inhalable Isofluorane at 2-2.5%. Post-surgery rabbits were given a second dose of buprenorphine hydrochloride as well as two doses the following day as a post-surgical analgesic.

The DrillSx was done unilaterally on the right hind limb of the rabbits. An incision was made on the lateral aspect using a 15 blade of the joint in-between the patellar tendon and extensor tendon. The fat pad was then deflected to access the joint. Eyelid retractors were used to

open the joint and allow access to the intra-condylar notch of the femur. Using a drill guide two holes were drilled into the femoral notch in a location free of cartilage approximately 7 mm apart to a depth of 10 mm into the marrow cavity with a 1.1 mm diameter surgical drill bit (ECT® internal fracture fixation drill, Zimmer, Warsaw, IN, United States of America) attached to a Dremel tool (Robert Bosch Tool Corporation, Mount Prospect, WI, United States of America). The joint and the skin were then closed using 4-0 Ethilon Cutting sutures (Ethicon Inc. Johnsons and Johnsons Inc., Skillman, NJ, United States of America) with continuous sutures, and a mattress suture at the end to release the stress on the skin.

Prior to surgery the rabbits were divided into two groups; feG treated (feG) and Saline controls (Saline), and were euthanized at 72 hours post-surgery with an overdose of sodium pentobarbital (©Euthanyl, Bimeda Group, Dublin, Ireland) IV.

Three rabbits received treatment with the tripeptide feG. The peptide was prepared by dissolving 1mg of feG in 1 ml distilled water, and then separated into 250 µl aliquots. The aliquots were then reconstituted to 2.5 ml of feG solution at a concentration of 1000 µg/kg treatments (reviewed in (412)). The rabbits were treated IV with a 500 µl/kg dose of feG solution 5 minutes prior to surgery. 72 hours post-surgery the rabbits were euthanized with an overdose of sodium pentobarbital (©Euthanyl, Bimeda Group, Dublin, Ireland) IV.

The other three control rabbits undergoing surgery were treated with sterile saline IV at a dose of 0.5 ml/kg (total ~3ml). They received an initial does of saline 5 minutes prior to surgery and a second dose 12 hours after surgery. Rabbits were euthanized 72 hours after surgery with an overdose of sodium pentobarbital (©Euthanyl, Bimeda Group, Dublin, Ireland) IV.

The remaining three rabbits served as age-matched non-operated controls (Ctl).

5.2. Tissue Collection

After the animals were euthanized, the joints were examined and photographed and the joint tissues were collected: synovium, fat pad, and cartilage (patellar, femoral groove, medial femoral condyle, lateral femoral condyle, medial tibial plateau and lateral tibial plateau). Portions of each of the tissues were flash frozen in liquid nitrogen while the remaining portions were embedded in Optimal cutting temperature (OCT) compound in cryo-molds (Tissue-Tek®, Sakaura Finetek, Torrance, CA, United States of America). The molds were immersed in cold isopentane/methylbutane (≤ -50° C) with care not to disrupt the tissue orientation. Additional synovial fluid was aspirated after a 1 ml sterile saline lavage was injected into the joint. Care was taken to ensure a minimum of 1 ml of synovial fluid was aspirated.

5.3. Histological Analysis

Cartilage was sectioned at -20° C at a thickness of 5 µm. Tissue blocks frozen in OCT compound were mounted onto a chuck with fresh OCT compound. Once the tissue was frozen on the chuck (approximately 3 minutes) the superficial layers of the block were shaved off until the tissue was exposed. The block was then faced by taking 10 to 20 5 µm sections (total 50-100 µm). Five to ten serial sections were then taken of the beginning of the block, then another 50-100 µm were removed and another set of five to ten serial sections were taken from the middle of the block, then a third set of 50-100 µm were removed and a final set of five to ten serial sections were taken from the rest of the block. Three sections of cartilage (patellar, femoral groove, medial femoral condyle, lateral femoral condyle, medial tibial plateau or lateral tibial plateau).

5.3.1. Safranin-O/Fast Green

Proteoglycans, cellularity, chondrocyte cloning and structure of cartilage was assessed using a Safranin-O/Fast green stain on sectioned tissue. Slides containing sections of cartilage (six locations collected) were hydrated through two changes of 100% ethanol, one change of

95% ethanol, one change of 70% ethanol, and one change of water. Slides were stained for 20 minutes in Gills II hematoxylin, and then washed in running water for 15 minutes. Slides were then stained for 5 minutes in fast green. Slides were placed in 1% acetic acid for 1 minute to decolour and the quickly rinsed in distilled water. Slides were further stained for 2 minutes in safranin-O, and dehydrated rapidly in a quick dip in 70% ethanol, followed by a quick dip in 95% ethanol and 3 minutes in two changes of 100% ethanol. Slides were cleared with Slide Brite™ (Sasco of Georgia Inc., Albany, GA, United States of America) and mounted with Permount (Fisher, Hampton, NH, United States of America).

Slides were examined with a Zeiss AxioPhot microscope (Carl Zeiss Inc., Thornwood, NY, United States of America). The slides and images were blinded and the sections were graded using a modified Mankin score (413), Appendix 3 Table 1.

5.4. Molecular Analysis

The synovium and fat pad were examined for mRNA expression of IL-1β, IL-6, IL-8, TNFα, and MCP-1 using real-time reverse transcription-polymerase chain reaction (qPCR) run in duplicate. (Appendix 3 Table 2 for primer sequences).

5.4.1. RNA isolation and quantification

Samples were powdered up with a dismembrator (Mikro-Dismembrator S, B. Braun Biotech International, Allentown, PA, United States of America) at 2600 RPM for 45 seconds and 0.3ml 100% ethanol/1 ml Trizol (Invitrogen, Carlsbad, CA, United States of America) was added to separate the DNA, mRNA and protein in the sample. DNA was quantified with PicoGreen on a plate reader (PerkinElmer, Waltham, MA, United States of America). The Trizol tissue mix was then transferred to a 1.5 ml Fisher tube (Fisher, Hampton, NH, United States of America). The total RNA was then extracted from the tissue using Qiagen RNeasy kit (Qiagen Sciences, Valencia, CA, United States of America). During isolation the samples were treated

with DNase-1 (Qiagen Science, Valencia, CA, United States of America) according to the manufacturer's guidelines to ensure there was no DNA contamination in the RNA preparations. Total RNA was quantified using SYBR green II fluorescent RNA dye (Bio-Rad, Hercules, CA, United States of America) on a plate reader (PerkinElmer, Waltham, MA, United States of America) with excitation and emission wavelengths of 468 nm and 525 nm respectively. The total RNA was quantified from a standard curve analysis (Workout 2.5, Dazdaq Ltd., Brighton, East Sussex, United Kingdom).

5.4.2. Real-time reverse transcription-polymerase chain reaction analysis (qPCR)

Total RNA was reverse transcribed to create a single stranded cDNA using Qiagen Omniscript RT kit (Qiagen Science, Valencia, CA, United States of America). Rabbit primers were designed and validated for the target mRNAs mentioned above (summarized Appendix 3 Table 2) as previously described (317, 414, 415). A PCR reaction mixture containing 12.5 µl of SYBR green supermix (Bio-Rad, Hercules, CA, United States of America), 3.5 µl molecular biology water, and 0.75 µl of each forward and reverse primer was used with 7.5 µl reverse transcriptase for each reaction. During amplification and detection an iCycler Thermal Cycler (Bio-Rad, Hercules, CA, United States of America) was used, validated via inspection of the melting curve for non-specific peaks. iCycler iQ Optical System software version 3.0a (Bio-Rad, Hercules, CA, United States of America) was utilized to quantify the results and the level of each target gene expression was normalized to 18S mRNA as an internal loading standard. The results are presented as fold change.

5.6. Statistical Analysis

Statistical analysis of histology was performed with a univariate analysis ANOVA plus Bonferroni post-hoc. qPCR differences between groups was performed with an analysis of variance (MANOVA) plus Bonferroni post-hoc using IMB® SPSS® Statistics 19 (IBM®

SPSS®, IBM® Corporation, Chicago, IL, United States of America). A Kruskall-Wallis test was used to verify the results.

6. RESULTS

During the course of the study animals appeared healthy, they were active in their cages and they ate and drank normally. Surgical animals regained baseline activity moving around cage within 6 hours after surgery (not quantified). There were no gross adverse effects seen with feG treatment.

6.1. Gross and Histological Data

There was minimal gross damage in the Saline and feG groups as seen in previously described studies. Some fibrillations and discolouration was present on the tibial plateaus and 2 of the six animals had small osteophytes along the femoral groove. These gross observations were consistent with the Mankin scores calculated histologically. There were no significant differences between Mankin subscores or locations therefore samples were pooled and summed. Surgical animals (Saline and feG) had higher mean Mankin scores that non-operated controls; however, these changes were not significant ($p>0.05$). The right limbs of Saline and feG also had higher mean Mankin scores than contralateral limbs ($p>0.05$). Figure 1.

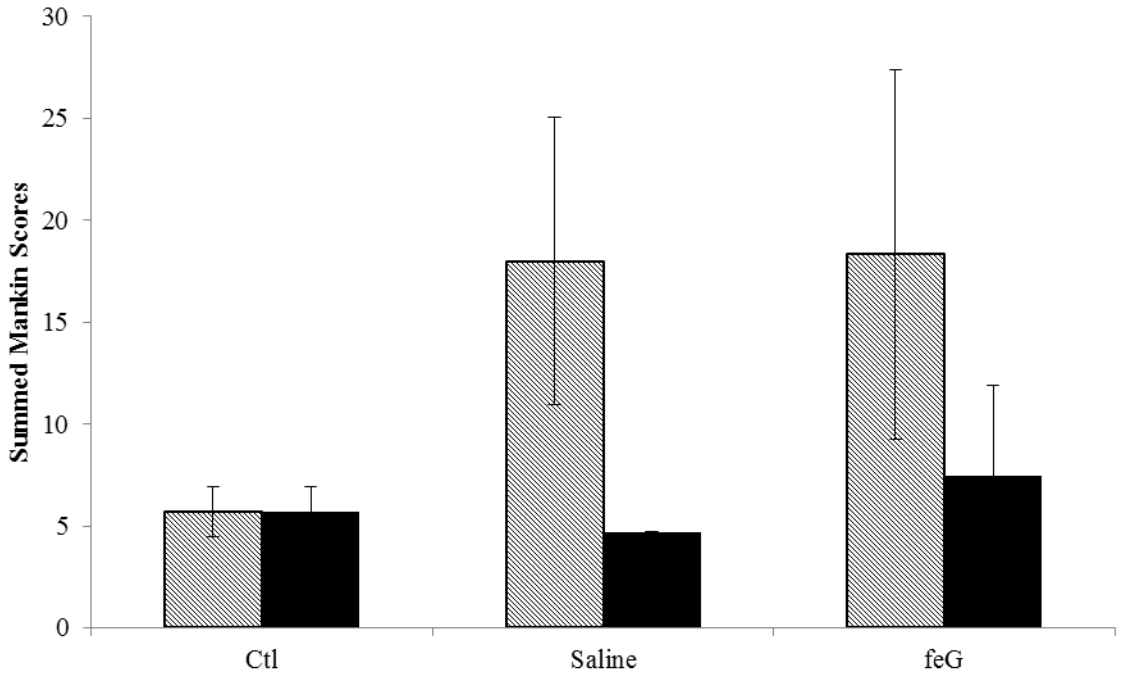
6.2. qPCR

There were no differences in expression of any of the markers in the fat pad (Figure 2a) or synovium (Figure 2b) between groups ($p>0.05$). TNFα, MCP1, IL-6, IL-8, and IL-1β were unchanged with feG treatment.

7. FIGURES

7.1. Figure 1 Cartilage histology

Figure 1. Summed mean Mankin scores. There were no significant differences ($p>0.05$) across groups or between limbs. Hatched boxes are right experimental limb and solid boxes are left contralateral limb. n=3/group.


Summed Mankin Scores
30
25
20
15
10
5
0
Ctl
Saline
feG

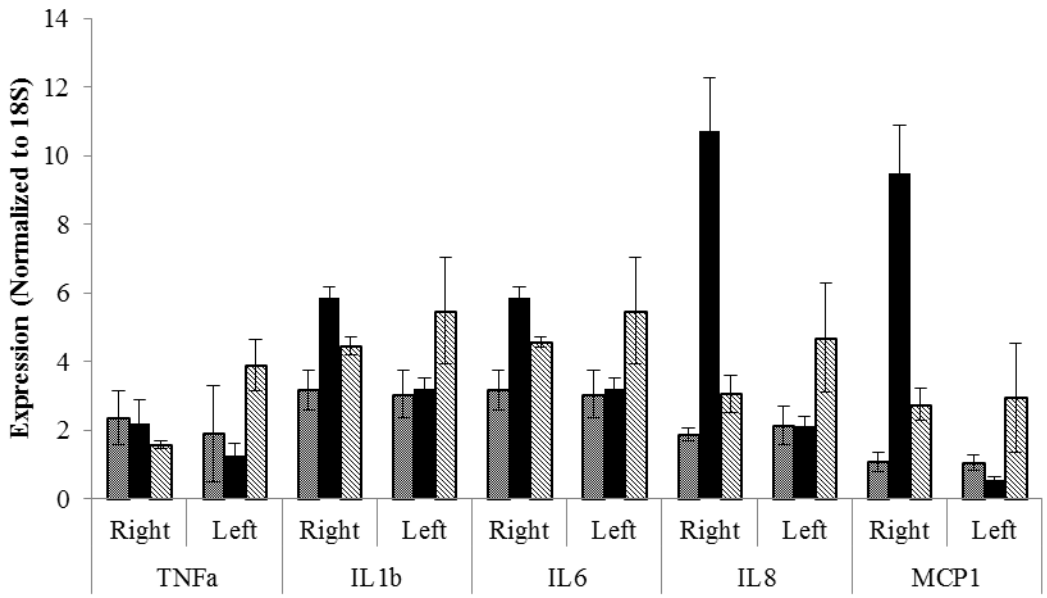
7.2. Figure 2 Fat pad and Synovial qPCR

Figure 2. a) There were no significant differences in expression of TNFα, IL-1β, IL-6, IL-8 or MCP1 in the fat pad ($p>0.05$).

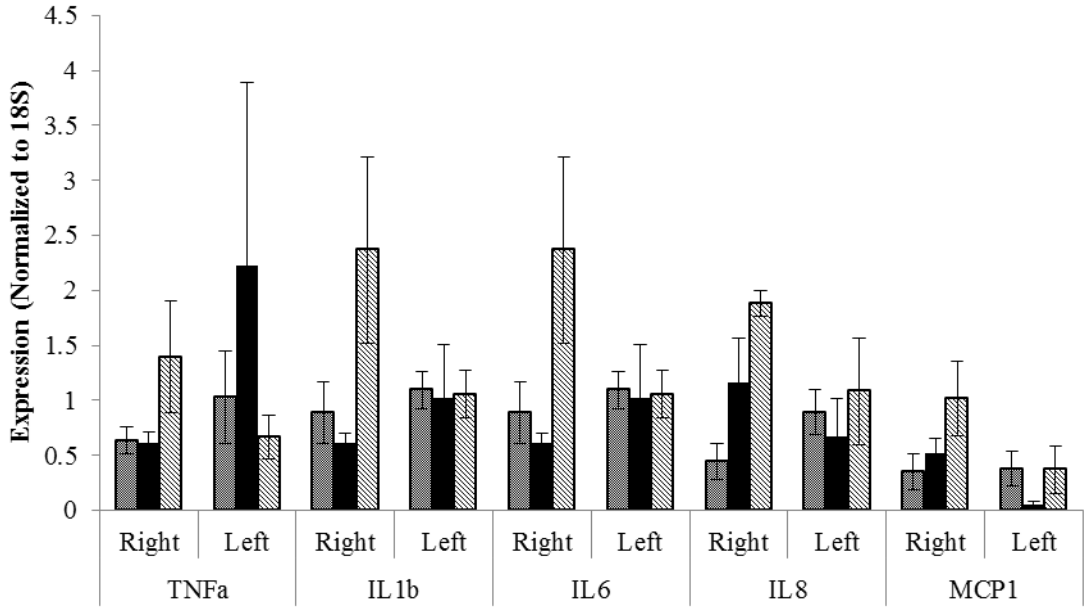
b) There were no significant differences in expression of TNFα, IL-1β, IL-6, IL-8 or MCP1 in the synovium ($p>0.05$).

Stippled boxes are means of non-operated controls, solid boxes are saline treated and hatched boxed are feG treated. Mean $\pm$ SD; n=3/group.

a)


Expression (Normalized to 18S)
14
12
10
8
6
4
2
0
Right
Left
Right
Left
Right
Left
Right
Left
Right
Left
TNFa
IL1b
IL6
IL8
MCP1

b)


Expression (Normalized to 18S)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Right
Left
Right
Left
Right
Left
Right
Left
Right
Left
TNFa
IL1b
IL6
IL8
MCP1

7. DISCUSSION AND CONCLUSIONS

This study is the first to use the tripeptide feG in a model of PTOA. Unfortunately results did not show any change in inflammation or prevention of very early cartilage damage with the use of IV feG. feG has been shown to alter inflammation in various models by interfering with cell adhesion; as synovial fibroblasts induce leukocyte adhesion in OA (410) it is reasonable to conclude that if this could be inhibited with feG inflammation, that some cartilage damage might be prevented in a rabbit model of PTOA. I hypothesized that feG would prevent cell recruitment to the joint and therefore inhibit inflammation and later cartilage damage. There were no significant differences in cartilage Mankin scores between groups and no differences in the expression of inflammatory markers between groups. Surgical limbs (right feG and saline treated) while not significant had higher Mankin scores, suggesting that there was some early cartilage damage with surgery which was not prevented with feG. I suspect there are a few possible reasons why feG failed to prevent inflammation and cartilage damage.

There are many possible explanations as to why feG was ineffective in this model of PTOA, including the possibly the dosage of feG was inappropriate; the doses used were based on the suggestion of Dr. Davison as per previous studies (403). However, none of the studies were in rabbits or for the treatment of chronic diseases. The joint is a unique isolated organ with limited blood supply; therefore systemic treatments do little to nothing to modify disease. As feG was administered IV and not intra-articular it is feasible that it failed to reach its target at the knee joint. Another possibility is that a single treatment was ineffective in preventing inflammation. The majority of studies done with feG showed effects in leukocyte recruitment after a single oral or IV dose of feG (395-398, 402, 403, 405, 407, 408), and one study used multiple IV doses in preventing leukocyte infiltration in a rat model of spinal cord injury (399). Therefore it is unclear if multiple or higher doses of feG would have had any effect in this model

of PTOA. Finally, previous studies have shown that cartilage damage worsens over time (349), so possibly rabbits were euthanized too early after treatment to show significant changes. However, this is unlikely as feG and Saline treated animals had worse scores in their surgical limbs after 72 hours.

A second consideration as to why feG was ineffective in this model of PTOA is that the tripeptide targets the wrong cells and the improper mechanisms for the development of OA. feG has been demonstrated to be a potent inhibitor of PMN adhesion and function via modulation of co-stimulatory molecules (401, 416). Many studies have investigated cell types in OA; Mϕ and T cells are the most abundant with Mast cells and B cells in lower numbers (review in (417). Neutrophils were almost never found in OA tissues (review in (417). Several studies using feG have prevented or decreased the recruitment of PMNs (395, 397, 402, 408) or eosinophils (405) without any effect on Mϕ. However, three studies feG inhibited Mϕ recruitment in pulmonary inflammation (403), intestinal motility (396), and most similar to the current model a spinal cord injury model (399). Mϕ numbers and infiltration is increased in OA synovial tissue (417-419); the number of Mϕ were not evaluated in the current study nor were leukocyte recruitment mediators. However, presuming Mϕ recruitment was inhibited other cells are also involved in OA and maybe compensating for the decrease in Mϕ. Unfortunately, while in future this would be beneficial, I was unable to conduct leukocyte recruitment studies with our current technology. In addition it is unclear in the aforementioned studies if decreased Mϕ numbers is primary or as a response to the inhibition of PMNs and its chemokines.

In conclusion, using the method and dose described above, feG was unable to prevent early joint inflammation and early cartilage damage in my rabbit model of PTOA. The mechanisms of feG in other models of OA need further investigation, as current studies have been limited to allergy, anaphylaxis and bacterial induced inflammation. While synovial and fat

pad inflammation are apparently important in the pathogenesis of PTOA, feG may not target the correct mechanism early in this disease. Further study in the mechanism of feG must be elucidated and direct measures of cell recruitment in this and other feG treated models of OA will have to be done to determine if there is any role for feG in the treatment or prevention of PTOA.

Chapter 6. OVERALL DISCUSSION

1. SUMMARY

The research presented in this dissertation represents an examination of the impact of inflammation on the development of post-traumatic osteoarthritis (PTOA) in the rabbit knee. By developing a model of PTOA and characterizing the response of joint tissues to various anti-inflammatory strategies, these studies offer a unique avenue by which to investigate the pathophysiology of post-traumatic OA (PTOA) and its potential prevention in this aniaml model. They clearly suggest a pivotal role for inflammatory stimuli in the development of cartilage damage and PTOA.

My first set of experiments (Chapter 2) involved the development of a novel animal model of PTOA, investigating the effect of drilling two small intra-articular holes in the intercondylar notch on subsequent joint inflammation and specifically to the articular cartilage of the knee of adult New Zealand White rabbits (349). Drill hole surgery resulted in a transient increase in a group of cartilage markers (MMP13 and TGFβ) and synovial inflammatory markers (IL-1β, IL-1Ra, IL-6, and IL-8). Most importantly, as compared with sham and normal controls, drill hole injuries reproducibly resulted in generalized cartilage damage in the injured joint, which progressively worsened over time. Unlike previous models (154, 294, 297, 302), this model demonstrated that PTOA can be caused without toxic biochemical induction of chondrocyte damage or inflammation, or by mechanically destabilizing the knee joint.

The response of the injured joint to glucocoticoid treatment was examined in the second study (Chapter 3) in which the impact of high dose glucocorticoid (dexamethasone) treatment on inhibiting joint inflammation and preventing articular cartilage was studied. Here I found glucocorticoid-mediated mRNA expression in the synovium and fat pad had a distinctive pattern. Dexamethasone inhibited some genes responsible for joint inflammation, scar formation/fibrosis

and cartilage degradation (IL-1β, MMP3, MMP13, IL-8, and collagen I). However, protein analysis with immunohistochemistry nonetheless demonstrated an up-regulation of some inflammatory proteins (IL-8, IL-1β) after surgery and only a trend for them to decrease after treatment with glucocorticoids. In addition, western blot data corroborated the decrease in IL-8 in the synovium, and also showed lower levels of IL-6 and IL-1Ra plus a decreased ratio of IL-1β/IL-1Ra in both the synovium and fat pad. Glucocorticoids also increased synovial and fat pad protein levels of TGFβ but most importantly, it supressed gross and microscopic cartilage damage. I can thus conclude that in this model, dexamethasone did reduce joint inflammation after injury and it did decrease cartilage damage. This suggests that early administration of dexamethasone after a joint injury may prevent later cartilage damage and joint degeneration. The effectiveness of dexamethasone given intrarticularly also further supports the notion from my first study, that my model of PTOA induces cartilage damage via biological changes after injury, whether from substances leaching out of the bone, inflammation during healing, or from some unknown immune reponse to the bone injury.

The next study, using my model of PTOA, focussed on the potential role of IL-1β in the pathogenesis of OA (Chapter 4). This study examined the use of intra-articular IL-1Ra injections to target and inhibit the IL-1β inflammatory pathway. IL-1Ra treatment led to improved histopathological scores but it did not eleminate all grossly observed joint damage. Interestingly, while no differences were seen in mRNA expression in markers of synovial inflammation, protein levels demonstrated a marked decrease in IL-1β, IL-6, bFGF, and most relevent, a decrease in the ratio IL-1β/IL-1Ra with IL-1Ra treatment. Changes in fat pad mRNA and protein showed an interesting pattern with increased levels of IL-1Ra expression and protein with treatment, while bFGF expression increased and protein levels decreased. There was also an increase in IL-6 protein and a decrease in IL-8 protein after treatment. Similar to the synovium,

both expression and protien levels of IL-1β/IL-1Ra decreased with treatment. In addition to changes in inflammation with treatment, there were increases in expression of genes responsible for cartilage regeneration and protection. While some changes were observed both at the molecular level and histological level with IL-1Ra injections, these were inconsistent suggesting that the inflammatory process in PTOA is complex and requires a multi-treatment approach.

The final study examined the short-term use of IV administration of the tripeptide feG (Chapter 5). feG was administered prior to surgery with the hope that it would prevent leukocyte recruitment to the site of injury and thus prevent damage. Despite positive outcomes in other animal models demonstrating feG inhibited leukocyte recruitment in anaphylaxis and acute inflammatory states, my results did not show similar benefits in the outcomes that studied at 72 hours post-injury in my model of PTOA. I observed no down-regulation of inflammatory mediators (TNFα, MCP1, IL-6, IL-8 or IL-1β) with treatment and no chondroprotective effect at the articular cartilage at that interval. I noted that there are several possibilities as to why feG failed to prevent these very early changes of PTOA in my model; for example, feG may workvia a mechanism that is irrelevant in this disease, or the route of administration, dosage, or timing, in the rabbit model was inappropriate to prevent inflammation.

The major findings are summarized below and then further discussed:

1. Drilling holes in the knee joint of the adult rabbit induces early and progressive cartilage damage, for up to one year after injury,consistent with the development of PTOA. This suggests that when gross changes in joint biomechanics are eliminated, biological joint changes (inflammation) appear to be capable of driving the development of some OA in this model.
2. Glucocorticoids are a potent inhibitor of joint inflammation and can prevent the development of cartilage damage in this model. However, systemic consequences of the

very high and repeated doses used in this study mitigate their effectiveness for clinical treatment at this point.

3. Inhibition of the IL-1β inflammatory pathway provides only partial joint protection altering aspects of joint inflammation (IL-1β/IL-1Ra, IL-6 and bFGF) in this model. While treatement with feG did not prevent joint inflammation or cartilage damage in this model.

2. PTOA IS A COMPLEX DISEASE WITH MULTIPLE THEORIES OF PATHOGENESIS. A COMBINATION OF GENETICS, MECHANICS, BIOLOGY AND LUBRICATION PROPERTIES MAY BE CONTRIBUTING TO THE DEVELOPMENT OF PTOA.

Despite decades of research the pathophysiology of PTOA is still unclear and there are various possible mechanisms underlying its development. Only 50-60% of individuals develop OA after a joint injury (153); it is unclear why the other injured individuals do not develop OA. It is possible that underlying genetics are protective for some and harmful for others leading to this varied result. IL-1β and IL-1Ra polymorphisms have been associated with knee OA (174, 175). Single nucleotide protein rs1433833 on the 3' untranslated region of growth differentiation factor 5 (GDF5) has been associated with OA (420-422). GDF5 is a member of the TGFβ family and helps in the development, maintenance and repair of synovial joints (423). Genome wide associated studies have found five significant single nucleotide protein loci associated with OA: chromosome 3 rs11177 coding for nucleostemin, chromosome 6 between FILIP1 and SENP6, chromosome 12 close to KLHDC5 and PTHLH and CHST11, and the FTO gene on chromosome 16 (424). Further studies have also identified rs2615977 in COL11A1 coding for the α1 polypeptide chain of collagen XI in association with OA (425). These studies are still very preliminary and are all in populations with primary OA. Therefore it is difficult to ascertain if similar genetic variances would be found in populations with secondary OA.

If genetic variability in patients is not the primary contributor to the development of secondary OA, it is possible that post-injury joint mechanics is the reason why only some of the population gets OA after an injury. Not all patients require surgical intervention after an ACL rupture, this population is often referred to as 'copers' while the remaining population is referred to as 'non-copers'. Biomechanical studies have shown that 'copers' walk differently than 'non-copers', where 'non-copers' have significantly greater knee extensor moment and shear force at

any given point in the gait cycle compared with ‘copers’ (426). This likely has implications for joint stability, but it remains unclear whether this has an impact on disease development. In addition, regardless of whether a patient is a ‘coper’ or ‘non-coper’ there appears to be a similar risk of developing OA (23, 160, 427, 428). Based on the literature it is still unclear how or if altered joint mechanics leads to OA post-injury. For a more thorough discussion on joint mechanics in ACL injury and reconstruction see below.

It is possible, therefore, that change in joint biology (synovial and fat pad inflammation) is the main contributor to the development of PTOA. Inflammation has been implicated in having a pathological role in both primary and secondary OA. Based on the current studies inflammation appears to cause PTOA without gross biomechanical or genetic variations. In my studies I was able to cause OA in genetically similar animals and without obvious disruption of joint mechanics. As I eliminated these variables in the studies presented in this dissertation, it makes it unclear in human populations what impact genetics and mechanics contribute to OA. However, in order to determine the components of each risk factor we must study them individually so in future we can create more complex models to combine multiple factors.

It is likely that the development of PTOA is multifactorial and is caused by a combination of genetic variability, changes in joint mechanics, post-injury joint inflammation, and changes in lubrication (discussed below). Inflammation also likely has other interactions with joint biology and mechanics, such as joint lubrication and cartilage wear (not investigated in this dissertation.

3. JOINT INJURY RESULTS IN BIOMECHANICAL CHANGES. AN ACL INJURY CAN CAUSE PROFOUND OA POST-INJURY.

While this dissertation has demonstrated that inflammation is part of the development of PTOA, in human injuries gross mechanical joint changes with resulting changes in joint contact stresses and stress redistributions may also contribute to PTOA. My model enabled me to isolate inflammation from gross mechanical changes after injury; however, in humans injuries (ACL being the most common) these cannot be isolated. An interplay between mechanical and cytokine mediated pathways are critical in the development of cartilage degradation post-injury (429).

It is possible that a combination of instability and inflammation result in worse cartilage damage or more severe OA. ACL injuries result in immediate abnormal motion (158, 159) and instability with activities of daily living. The ACL is important for tibiofemoral kinematics, providing both rotational stability and anterior-posterior translation stability. After an injury this tibiofemoral motion is changed; exactly how it is changed is still up for debate. Some studies suggest the tibia is in a position of greater internal rotation and posterior translation in stance phases of gait (158) and at low flexion angles (430) and with flexion-extension motions (431) after and ACL injury. With lunging activites there appears to be medial impingement of the tibial spine by the medial femoral condyle (159). These studies often fail to agree because they are testing different activities; despite this there are some clear differences in joint biomechanics after an injury. These biomechanical changes ultimately result in altered joint loading of the articular cartilage. Immediately after injury there are decreased forces on both medial and lateral compartments of the joint during gait (432, 433). Both peak knee compression forces and peak knee shear force are significantly reduced during gait (426). Unloading the knee results in cartilage atrophy and death.

These studies raise a major question. If ACL reconstruction is done early after injury will biomechanics be restored? In a recent study done in our lab, we found that in sheep even with an 'ideal' reconstruction of the ACL joint using the native ACL reattached immediately, mechanics were not restored to pre-injury gait kinematics (380). Similarly patient studies showed that ACL reconstruction failed to restore normal joint kinematics or medial translation (434). Interestingly after an ACL reconstruction peak-abduction moment is increased (435), which is the converse of what has been shown post-injury. It is likely that certain areas of the joint have increased dynamic loading where others have decreased dynamic loading both resulting in OA. From these studies the clinical relevance of these gait changes post-reconstruction is unclear. It is also not clear whether altered joint biomechanics lead to OA in these patients, or if other biological factors result in OA despite these minimal biomechanical changes. In addition the mechanical changes may perpetuate inflammation in the joint furthering the progression of OA. Hopefully by separating the biological joint changes after injury and reconstruction we may be able to elucidate both of their roles in the development of OA and target them appropriately in future to prevent OA.

Not only do joint mechanics play a role in post-injury disease, but tissue maturity may be important in healing and treatment strategies. It is generally thought that younger individuals heal faster than older individuals; however, immature patients have higher failure rates of ACL reconstruction post-injury than their mature conterparts. In order for successful ligament healing there must be adequate fibroblast numbers and activity. Cells from immature individuals and animals have statistically higher numbers and increased migration of fibroblasts in both intact and torn ACLs (436-438); these fibroblasts also grow faster than their adolescent and adult counterparts (437). In addition to cell numbers being greater there is significantly more collagen synthesis, water content, and reducible collagen crosslinks in immature ACLs (439). I also found

increased levels of collagen I, V, and VI with lower decorin expression (440). After an ACL reconstruction, grafts also remodeled faster in immature sheep when compared with adult sheep (441). There has unfortunately been no work demonstrating the mechanical changes in the ACL with maturation, therefore it is unclear if underlying ligament mechanics could be playing a role in higher ACL recontruction failure rates in pre-pubescent children. It is also possible that immature joints mount a greater inflammatory response as well predisposing the ligament to fibrosis and failure.

4. GLUCOCORTICOIDS ARE A POTENT INHIBITOR OF JOINT INFLAMMATION AND CAN PREVENT EARLY CARTILAGE DAMAGE POST-INJURY.

Glucocorticoids have been used for the treatment of various orthopedic and rheumatological conditions for over 60 years. Despite their widespread use, and analgesic effects very little research has been done on the impact glucocorticoids have on joint inflammation and articular cartilage with emphasis on modifying disease. Various adverse effects are reported with the use of glucocorticoids including avascular necrosis, tendon rupture, Cushing's syndrome, osteoporosis, weight gain, and infectious arthritis (442). The present study (Chapter 3) revealed that intra-articular dexamethasone not only inhibited inflammation but prevented cartilage damage after surgery. However, systemic effects were seen with the large doses being administered resulting in significant weight loss and gross organ damage. Therefore, the regime used in this study would be inappropriate for treatment. While the dose and frequency of glucocorticoids administered may not be appropriate for patient use, valuable information can still be garnered from these results. Gene-specific and protein changes due to glucocorticoids were seen in the fat pad, synovium and cartilage. Collagen and MMP genes showed a significant depression of mRNA after glucocorticoid treatment in the fat pad and synovium, similar to previous studies (350). Other genes and proteins, such as IL-1β, IL-8, IL-6 and the ratio of IL-1β/IL-1Ra in the fat pad and synovium were responsive to glucocorticoids. Most interesting was that cartilage damage was inhibited by glucocorticoids and glucocorticoid administration produced more growth factors, fewer degradative mediators, and fewer inflammatory cytokines. Macrophages likely are playing a big part of the development of PTOA, as they arrive to a site of injury after tissue damage with 48 hours and can remain in situ for months after an injury perpetuating inflammation and further damage.

Glucocorticoids have dose-dependent effects: low doses are chondroprotective (443) and high doses are chondrotoxic (366). The majority of these studies have been with betamethasone sodium phosphate or betamethasone sodium acetate, but a recent study demonstrated that 7 days of 1.2mg dexamethasone was also chondrotoxic *in vitro* (365). These studies have not been mimicked *in vivo* and I showed a chondroprotective effect in my model, leaving the effect of glucocorticoids on cartilage still controversial. By using glucocorticoids and inhibiting inflammation I have been able to demonstrate that inflammation is integral in the development of OA. While glucocorticoids should be used with care due to their potential chondrotoxic and systemic effects, I was clearly able to show that it was chondroprotective in this model. In light of the result it is a likely there is a need for targeted anti-inflammatories to prevent OA post-injury.

5. INHIBITION OF THE IL-1B INFLAMMATORY PATHWAY PROVIDES ONLY PARTIAL JOINT PROTECTION ALTERING ASPECTS OF JOINT INFLAMMATION (IL-1B/IL-1RA, IL-6 AND BFGF) IN THIS MODEL OF PTOA.

Inhibition of IL-1β with Anakinra (Chapter 4) influenced other inflammatory cytokines (IL-8 and IL-6) in the fat pad and synovium and increased bFGF and TGFβ expression from the articular cartilage. This resulted in partial cartilage protection; histopathological scores were lower after treatment but some gross damage was still evident.While possible inhibition of PMN recruitement with feG did nothing to prevent cartilage damage after surgery. As previously mentioned the mechanism of inflammation in PTOA is likely driven by Mϕ which release IL-1 and TNFα. Therefore by inhibiting IL-1 downstream to the influx and activation of Mϕ at the site of injury PTOA may prevent PTOA.

The results of this study raise three major questions. First, with a short half-life are there more effective delivery methods of IL-1Ra? Second, why was only partial joint protection acquired from Anakinra treatment? Lastly, would a combination of anti-inflammatories completely prevent PTOA?

As discussed previously Anakinra has a short-half life; pharmacokinetic studies have shown a serum half-life of 4 hours after intra-articular injection (286). Patients with RA that have failed other treatments require daily injections for benefit. Gene transfer of IL-1Ra has had success in suppressing experimental arthritis in various animal models (273, 281, 282, 284). All but one of these studies used a surgically induced model of OA, similar to the current study. Unfortunately after a small series of studies from 20 years ago there were no further studies targeting other delivery methods of IL-1Ra. Despite this concern several clinical trials have been done investigating the safety of anakinra in OA (285, 286), and a pilot randomized control trial has shown reduced knee pain and improved functional scores with an injection of anakinra

within one month of an ACL injury. A new clinical trial (NCT01692756) is currently beginning recruitment of patients to trial anakinra treatment 1-2 days, 12-14 days, 2 weeks post-op, 4 weeks post-op and 6 months post-op after an ACL tear. The researchers plan to evaluate not only functional scores (KOOS), but to measure inflammatory cytokines (IL-1α, IL-1β, collagen I, collagen II, cartilage oligomeric protein, GAG, and xanthine oxidase and meniscus and cartilage metabolism, and oxidative stress. These results should prove interesting.

The current study was only able to partially inhibit inflammation and prevent cartilage damage. The inflammatory process is very complex with multiple compensatory molecules allowing for redundancy. IL-1β is unique from other pro-inflammatory cytokines, such as IL-6 and TNFα as it follows a non-conventional route of protein secretion (444) which is promoted by extracellular ATP. The NALP3 inflammasome is known to be central in the processing of IL-1β and release. Therefore it is possible that IL-1Ra does not target high enough up the cascade, and therapies should target NALP3 and its activating stimuli. It is also possible that as IL-1Ra only inhibits the inflammatory cascade signaled by IL-1β, other pro-inflammatory cytokines are still active in driving joint inflammation and OA.

With the idea that IL-1Ra may not be broad enough to target the complex process of post-injury inflammation, but glucocorticoids are too broad with too many adverse effects, possible combination targeted anti-inflammatory therapies will be beneficial. None of the other current anti-inflammatory therapies have been tested in combination with each other; however, there is another potential treatment that seeks to combine anti-inflammatory cytokines and growth factors to treat musculoskeletal conditions. Autologous conditioned serum (Orthokine) is thought to contain several beneficial proteins including IL-1Ra. By exposing whole blood to chromium sulfate the production of IL4, IL-10, IL-1Ra, bFGF, hepatocyte growth factor, and TGFβ is stimulated. Orthokine has been shown to result in significant improvement in cartilage scores in

animal models of OA (445, 446). It also improves clinical WOMAC scores after 2 years of follow-up in patients with OA (447) and post-ACL reconstruction (448), with no adverse reactions (449). None of these studies has evaluated whether orthokine is protective of cartilage or modifies or prevents disease. It would be worthwhile investigating orthokine and comparing it to other treatment modalities in an *in vivo* model, such as my model of PTOA. It is clear that there needs to be more research on its efficacy not only to improve functional outcomes, but to modify or prevent disease.

6. INFLAMMATION LIKELY PLAYS A ROLE IN JOINT LUBRICATION RESULTING IN ALTERED JOINT LOADING.

Synovial fluid is made of various lubricating molecules produced by synovial cells and superficial zone chondrocytes. Normal synovial fluid is essential in maintaining articular cartilage and is chondroprotective (450). Lubricin/PRG4/superficial zone protein and hyaluronic acid have been identified as key lubricating molecules in the synovial fluid (451) and at the articular cartilage surface (452). Lubricin is a boundary lubricant mediating friction between articular cartilage surfaces. It is effective on its own, but with hyaluronic acid it reduces friction even further.

Lubricin expression is inversely correlated with OA severity (453), where OA synovial fluid is deficient in lubricin (454). This lack of lubricin decreases boundary lubrication of the articular cartilage by increasing the coefficient of friction (453, 454). This is relevant as friction and wear at the articular cartilage surface are highly associated (455). This raises the question of whether OA changes lubrication properties or do changes in lubrication properties lead to the development of OA? It is likely cyclical, where initial changes in lubricin and hyaluronic acid wear the articular cartilage and as articular cartilage wears the synovial fluid composition changes further leading to more wear and so on. After a joint injury there are immediate changes in both hyaluronic acid and lubricin. Hyaluronic acid levels in the synovial fluid decrease after injury and the coefficient of friction increases (456). Lubricin levels, however, increase early after a joint injury and return to baseline levels after a few days. While the lubricin level changes are transient, there is a long term increased coefficient of friction at the articular cartilage surfaces (457).

The findings of these studies raise two main questions. First, what leads to the changes in synovial fluid lubricating molecules after an injury? Second, why do intra-articular injections of

hyaluronic acid fail to modify OA? Several studies have linked joint inflammation and related growth factors with changes in lubricin levels. Patients with traumatic synovitis have decreased synovial fluid lubricating properties (458). After an ACL rupture, patients have increased levels of TNFα, IL-1β, IL-6, procathepsin B, and neutrophil elastase in the synovial fluid, and both TNFα IL-1β levels have been correlated with decreased lubricin levels in the synovial fluid (459, 460). TGFβ up-regulates lubricin by surface chondrocytes (461-466) in all joint compartments (467), and infrapatellar fat pad progenitor cells (468), and synoviocytes (469). Oncostatin M, a pleiotropic cytokine of the IL-6 family also increases the accumulation of lubricin by surface chondrocytes (466), while bone morphogenic proteins (BMP2,BMP4, and BMP7) increase the accumulation of lubricin by chondrocytes, fat pad progenitor cells, and synoviocytes (464, 468, 469). While these growth factors induce lubricin production and expression, other cytokines reduce lubricin levels. IL-1 (IL-1α and IL-1β) reduces the expression and secretion of lubricin from surface chondrocytes (461, 463, 466, 467), as does TNFα and retinoic acid. In addition to these studies anti-TNFα Etanercept therapy after an ACL injury significantly increases the lubricin concentration in synovial fluid resulting in decreased GAG release and chondroprotection (460).

These studies all demonstrate that various pro-inflammatory mediators decrease lubricin synthesis and release from explanted tissues, suggesting inflammation may drive joint lubrication changes and further cartilage wear and that without targeting the underlying cause, intra-articular injections of lubricating molecules on their own will be ineffective in preventing OA. However, this does pose some interesting treatment strategies for combination therapies. This invites further investigation into the *in vivo* relationship between inflammation and lubrication in my model of PTOA.

Chapter 7. LIMITATIONS

Several limitations and assumptions must be taken into account when interpreting the results in this dissertation. First, sample sizes for all groups were small, but large enough to see differences in histological and mRNA results. In addition the animal model used in this dissertation was the New Zealand White, which is only a partially inbred strain of rabbit and therefore not as homogeneous as other animal models. Also there are some clear mechanical differences in rabbits and humans as one is a quadruped and the other a biped. However, as stated previously the rabbits are a good model for studying orthopedic injuries and OA.

Also, the molecular data presented in this thesis was analyzed for the most part using real-time quantitative PCR (qPCR). There are several limitations of qPCR for the analysis of tissue samples. Some studies have also demonstrated that a single housekeeping gene, as used in these studies is insufficient for normalization (470). As with other systems, there are limitations in detection and analysis. However, qPCR has been shown to be more accurate and less variable than its older counterparts. It has also been validated for cytokine analysis in human and mouse inflamed tissues (471). Western blot analysis of cytokines and growth factors was done to confirm qPCR results (Chapter 3 and 4), and previous rabbit studies have also shown a good correlation between mRNA and protein (350, 472).

Other limitations were the dosages used for all treatments in these studies (Chapters 3-5). Corticosteroid doses were based on previous studies by Kydd *et al.* (350, 411, 472), IL-1Ra doses were based on human equivalent dosages and previous studies, and feG dosages were based on recommendations from Dr. J Davison. Dose studies for efficacy were not done and therefore dosages and routes of administration may not have been optimized in these studies and for the rabbit model.

The premise of this dissertation is that inflammation has been isolated from mechanics; however, due to the limitations of the animal model, biomechanics were not measured. Based on qualitative assessment of the rabbits after surgery their gait appeared unaffected. However, inflammation itself may lead to alterations in joint mechanics as per lubrication changes.

Finally, the markers chosen for analysis in this dissertation were chosen based on previous studies of the sheep model in our lab. Specific cell type analysis and a more thorough analysis of various inflammatory molecules and associated molecules would be beneficial for a more complete analysis of this model and subsequent treatment (see future directions).

Chapter 8. MAJOR CONCLUSIONS

These studies indicate that inflammation plays an important role in the development of PTOA after an intraarticular bone injury in rabbits. The joint is very responsive to inflammation; Synovial and fat pad inflammatory up-regulation stimulates cartilage changes including increased degradative markers and decreased chondrogenic markers. I was successful in developing a model of PTOA that does not cause gross biomechanical changes in order to tease out the effects of inflammation versus mechanical changes in the joint after an injury. This was proved by inhibiting cartilage damage with high dose glucocorticoids, which blocked inflammation in the joint after injury. However, the dosages of dexamethasone used in this thesis led to systemic effects including organ damage and weight loss. As such a more targeted approach to preventing inflammation is more appealing. By inhibiting the IL-1β pathway with anakinra I was able to partially alter inflammation in the synovium and fat pad most notably the ratio of IL-1β to IL-1Ra. This prevented histological cartilage damage despite some gross damage. Post-traumatic OA inflammation is complex, likely with redundancy and will need to be treated as such in the future using multipronged strategies for treating individuals after a joint injury.

I sought to separate inflammatory variables involved in PTOA from biomechanical changes after injury by creating this model. This has provided an exciting new avenue by which to study the inflammatory mechanisms involved in PTOA and prevent part of the pathogenesis of OA. Therefore in future after understanding inflammation in PTOA we should be able to target both post-injury inflammation and mechanical instability seen after injury.

Chapter 9. FUTURE DIRECTIONS

I have been able to create a novel model of PTOA in which biological joint changes drive cartilage damage. This has opened a new avenue by which to study the biology of joint injuries, including inflammatory mechanisms. In addition it has made it possible to investigate treatments targeted at post-injury joint inflammation in order to prevent PTOA.

The characterization of the changes in the joint tissues following injury reveals a complex inflammatory process driving post-injury cartilage damage and disease. Because of the multifactorial risks that lead to post-traumatic OA, it was important to create a model in which the biological components driving OA could be investigated. The findings of this dissertation indicate that post-injury/surgery joint inflammation alone can lead to cartilage damage and OA and that these changes are sensitive to glucocorticoid treatment likely inhibiting NFκB and post-transcriptional pro-inflammatory molecules (COX2, IL-1β, IL2, and others). Post-injury joint inflammation was also partially inhibited by IL-1Ra resulting in a chondroprotective effect. Further studies revealed that feG a tripeptide responsible for inhibiting neutrophil recruitment had no effect in my model, suggesting that inflammatory inhibition by IL-1Ra and glucocorticoids was not mediated through increased neutrophil migration to the site of injury.

To examine further the role of inflammation in this model and its relationship with cartilage damage a number of studies could be performed. First, feG studies failed to prevent joint inflammation and cartilage damage suggesting that leukocyte recruitement is not key in post-injury joint inflammation (Chapter 5). Analysing the cell populations involed in inflammation after surgery by measuring cell migration, recruitment, and proliferation would determine, first, if cell recruitment or local cell proliferation was up-regulated and contributing to OA. Second, macrophages, T cells, B cells and mast cells have all been identified in OA (417); however, very little has been done to characterize cell populations after injury and examine how

they change over time. Identifying the cell types present in various joint tissues would both increase our understanding of the pathophysiology of disease and lead to targeted prophylactic therapies. Additionally, analysis of cell types would help in understanding which cytokines, chemokines, proteases, and growth factors were released and from which tissues.

Since initial animal studies (Chapter 2) were small and much of the molecular data was not corroborated with protein analysis, future studies could be performed with an n=11 (power analysis) and correlating mRNA analysis to western blotting, immunohistochemistry, or ELISA. As several genes have been shown to have expression which does not correlate to post-transcriptional proteins, this study might be important for confirming true tissue results. In addition to correlating western blot data to PCR data, a more throrough study of cytokines, chemokines, proteases, and growth factors would be beneficial. The genes and proteins studied were based on previous studies from the lab and availablility of suitable anitobodies, therefore important factors may have been overlooked that would help explain the pathways of inflammation activated after injury. These studies may also benefit from metabolomic exploration, which can provide information on the physiology of the cells complementing genomic and proteomic data. Determining metabolites present after a joint injury helps determine the physiological impact of up-regulated or down-regulated genes and proteins.

While well studied, the rabbit model does have limitations when investigating immune responses. One of the challenges in studying complex biology in the rabbits is their relative heterogeneity. A second challenge is the availability of reagents, such as antibodies, the majority of which are currently made in rabbits. It is a challenge to find antibodies that will be specific for use in western blots, ELISA, and immunohistochemistry with rabbit tissue. A rat model would allow the surgery developed in this research to be replicated and would enable the study of the details listed above. There are several advantages of the rat model, its joint is large

enough that it is feasible to microdrill in the joint but small enough that the whole joint can be examined histologically. In addition, genetically identical animals are available, the rat has been used as a model of OA so it can be compared to other data in the literature, and biological methodology is more readily available for the rat.

The findings in this dissertation propose a role for inflammation in the development of OA by eliminating mechanical joint changes post-injury; however, I failed to demonstrate that there were actually no changes in biomechanics or loading of the joint after injury. In order to determine that my model develops PTOA by biological changes alone, it would be beneficial to do biomechanical studies post-surgery. We have a well established sheep model in which a highly sensitive spatial linkage system is used to collect realtime kinematic data (unpublished). In order to confirm that there are no mechanical changes after surgery, the current drill surgery could be transferred to the sheep model and both *in vivo* kinematics and tissue loads could be investigated with our six degrees of freedom robot. After determining that this model of PTOA eliminates mechanical joint changes, and establishing the inflammatory mechanism involved in the development of PTOA (discussed above), mechanical studies of an ACL deficient model might provide insight into the combination of mechanics and biology that lead to PTOA.

In order to further determine the benefit of IL-1Ra prophylaxis in preventing OA after an injury or surgery, it may be useful to try different treatment regimes and dosages of IL-1Ra. Also as previously mentioned, recombinant IL-1Ra has a short half life requiring an impractical and undesirable administration regime for patients. Therefore, other methods of delivering IL-1Ra could be more practical. Gene therapy showed some promise, but needs further investigation. The use of autologous conditioned serum might show promise in my model as it combines various anti-inflammatory proteins together and could provide complete inhibition of joint inflammation. Treatment with orthokine might yield a greater protective effect, and would be

worth doing in either the rabbit model or the rat model. Another valuable study to investigate OA prevention is by the use of siRNA. Several studies have used RNAi as a therapy in models of arthritis including knocking out various cytokines (473). Most recently Santangelo *et al*. (474) knocked out IL-1β expression/signaling in a guinea pig model of OA which decreased IL-1β, TNFα, and IL-8 for 180 days and increased TGFβ. It might be helpful to knockout IL-1β in a rat model of my model of PTOA to determine if post-injury inflammation is inhibited and long term chondroprotective effects are achieved. Plasmids can be optimized for rat short hairpin RNA sequences and functional, histological, and molecular outcomes can be measured to determine their efficacy in preventing PTOA.

Of interest would be the relationship between post-traumatic inflammation and joint lubrication. Adding lubricin and hyaluronic acid therapy to an anti-inflammatory could provide insight into this relationship and determine if there is a role for addressing lubrication supplementation in therapeutic prophylaxis after a joint injury. Additionally mechanical studies could be incorporated to investigate changes in boundary lubrication and the coefficient of friction at the joint surface to elucidate the inter-relationship of inflammation, lubrication and surface cartilage wear after an injury.

This model of PTOA has opened many new avenues by which to study joint injuries and the development of OA after an injury.

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APPENDICES

1. POST-NATAL MOLECULAR ADAPTATIONS IN ANTEROMEDIAL AND POSTEROLATERAL BUNDLES OF THE OVINE ANTERIOR CRUCIATE LIGAMENT: ONE STRUCTURE WITH TWO PARTS OR TWO DISTINCT LIGAMENTS?

1.1. Abstract

The human anterior cruciate ligament (ACL) is composite structure composed of two anatomically distinct bundles: an anteromedial (AM) bundle and posterolateral (PL) bundle. These two bundles display different tensioning throughout joint motion and thus may, in turn, have different functions and different molecular and ultra-structure. It is widely accepted that there are structural and molecular changes to the ACL throughout development and maturation; however, it is unclear whether these bundles are similar or distinct throughout maturation. The aim of the present study was to assess similarities and differences in the ultra-structure and molecular composition of the even more distinct AM and PL bundles of an ovine ACL during growth and development. In order to examine maturational differences, mature (3-4 year old) and immature (1-3 month old) Suffolk cross sheep were compared. Central portions of their isolated AM and PL bundles were analyzed with q-PCR for collagens type I, III, V and VI, decorin and biglycan, western blot for collagens type I, III, V and VI and decorin, and TEM to measure fibril diameters. Results showed that maturation alters the ACL; immature sheep have significantly more collagen synthesis, matrix activity and fibrillogenesis as seen by higher levels of collagen type V and collagen type VI mRNA expression and proportions of collagen type V and VI protein. Confirming fibrillogenesis, immature ligaments also have significantly smaller collagen fibril diameters than the mature adult sheep. Interestingly the PL band appeared to mature more slowly than the AM band, but despite different maturation rates, in the adult sheep

we detected no differences between the bundles. We conclude that despite having two bundles, the two anatomically distinct bundles of the sheep ACL should be considered as two parts of one structure.

1.2. Introduction

The anterior cruciate ligament (ACL) is a multi-fascicular structure whose individual bundles are each ensheathed in loose connective tissue (475). Whereas for many decades the adult human ACL was considered to be a single ligament (5), recent evidence suggests that it probably should be considered as a composite structure with two functionally and anatomically distinct bundles: the anteromedial (AM) and posterolateral (PL) fibrous bundles (476). Further, it has been noted that these bundles are distinguishable early during fetal development (477) with a well-defined highly vascularized, connective tissue septum dividing the two bundles of ACL (477). Recent studies using magnetic resonance imaging have also confirmed the double bundle anatomy of the ACL in many people, although distinct AM and PL bundles could be visualized only in about one-third of patients (478).

Functionally, the ACL provides stability to the knee by restraining anterior displacement of the tibia with respect to the femur (19) and by providing resistance to rotational loading throughout the range of motion of the knee (479). The fibres of the AM bundle tighten while the PL bundle is lax during knee flexion, whereas when the knee is extended the PL bundle is taut and the AM bundle is moderately relaxed (476). A precise understanding of the functional anatomy of the ACL and the roles of each band in carrying load at different joint angles is considered to be essential for successful surgical reconstruction, to recreate this normal tensioning pattern of both components of the ACL and thus for the long term success of surgical reconstruction (480).

Interestingly, while widely accepted that the human ACL is composed of two bundles, it is not yet clear whether these bundles are similar or distinct with respect to their molecular composition in adulthood. Water, collagens, and glycosaminoglycan (GAG) are known to be the basic components of the extracellular matrix of typical ligaments (481, 482). Collagens are the

principal structural constituents of ligaments with the type I collagen predominating and the rest a composite of collagen type III, VI, V, XI and XIV (483-485). There is a smaller proportion of equally important proteoglycans, with several types of proteoglycans being found in ligaments (486). Ligaments also show regional biochemical (487) and ultra-structural (488) variations and these matrix components do change with maturation. For example, in the maturing ACL, these changes include an increased proportion of matrix and collagen type III (484), but it also features decreases in: cell density, blood vessel density (489), expression of vascular endothelial growth factor isoforms (490), alpha smooth muscle actin (489); and glycosaminoglycan (GAG) content (484). Despite these observations of ligament matrix changes with maturation and the likelihood that functionally distinct bundles of the ACL will have distinct matrix composition, specific comparisons of the two bundles of the ACL during growth and development or at maturation have not yet been examined.

Individuals involved in sports are often subject to injuries to ACL. Surgical reconstruction is the recommended treatment for torn ACLs. There is increasing interest in comparing the efficacy of single-bundle versus double-bundle ACL reconstruction to repair the damaged ACL. As the primary aim of the reconstructive surgery is to recapitulate the original structure of the intact ACL, the debate over the best surgical reconstruction technique to achieve this continues. Furthermore, it would appear that the long term prognosis of even clinically successful ACL reconstructions is not ideal, with 40% of people developing osteoarthritis (OA). Therefore, it is important to have a complete understanding of the anatomy and molecular biology of the two bundles of the ACL to try to achieve better reconstruction of the ACL that does not result in OA in the long term.

For the past seven years, we have been using an ovine model of ACL injury and reconstruction to understand the implications of ACL deficiency to joint mechanics and to the

development of OA (154, 302, 305, 491). We have found that, as in humans, the ACL is a key structure whose deficiency leads to important kinematic abnormalities and to the development of early OA. The ovine stifle joint is widely used as a model to study the impact of joint injuries, develop new surgical techniques for repair of ligaments and for understanding the development of OA following ligament or meniscal injuries. The use of this model is based primarily on the assumption that the tissue response to the healing process is very similar to those in humans (492). The ovine ACL has two grossly distinct bundles that discretely split over the anterior horn of the lateral meniscus. However, it is unclear if these two bundles are structurally and molecularly identical. Given the high incidence of OA development in spite of successful ACL reconstructions it is important to understand the makeup of the two bundles completely to answer the basic but important question - is this one structure split into two identical parts or two distinct structures?

The aim of the present study was therefore to assess the morphological and molecular similarities and/or differences between the AM and PL bundles of the ovine ACL during its growth and development, to determine if it should be considered one structure or two structures. Our hypothesis was that due to distinct functional roles, the AM and PL bundles of the ACL will have distinct patterns in matrix composition and ultrastructure in immature and skeletally mature individuals thereby allowing us to define whether or not the ovine ACL should be considered a single structure with two parts or two distinct structures. We believe that the results of this study have important consequences for ACL reconstruction surgery.

1.2. Materials and Methods

1.2.1. Animal model and tissue harvest

The current study was conducted following approval from the Animal Care Committee at the University of Calgary in compliance with the Canadian Committee for Animal Care. Fifteen female Suffolk cross sheep were used; nine were skeletally mature (3-4 years old) and six were juvenile, skeletally immature lambs (1-3 months old). All animals were euthanized using Euthanyl (Bimeda-MTC, Cambridge, Ontario) administered intravenously. Following sacrifice, the two bundles forming the ACL— the AM and the PL bundles, were isolated and collected from the knees of juvenile and mature animals. At dissection, the central portion of each of the AM and PL bundles of the ACL tissue was isolated for transmission electron microscopy (TEM) and fixed in glutaraldehyde and Malonic buffer. The remaining AM and PL bundles of the ACL were snap frozen in liquid nitrogen and stored frozen at -80°C until further molecular and protein investigations were performed.

1.2.2. RNA extraction and quantification using qPCR

The AM and PL bundles of ACL were examined for mRNA expression of collagens; Collagen type I, Collagen type III, Collagen type V and Collagen type VI and the proteoglycans; decorin and biglycan (see appendix 2 Table 2 for primers) using real-time reverse transcriptase polymerase chain reaction (RT-PCR). Real time RT-PCR was performed using techniques previously described (317, 493). Briefly, total RNA was extracted from AM and PL bundles of ACL using Qiagen RNeasy kit (Qiagen Sciences, Germantown, Maryland), treated with DNAse-I and quantified. Total RNA was reverse transcribed to generate single stranded cDNA using Qiagen Omniscript RT kit (Qiagen Sciences, Gemantown, Maryland). Primers were created and validated for the target mRNAs above. A PCR reaction mixture containing BIO-RAD iQ SYBR Green Supermix (BIO-RAD, Hercules, California) and forward and reverse primer was used for

each reaction. iCycler Thermal Cycler (BIO-RAD, Hercules, California) was utilized during amplification and detection, and validated through inspection of the melting curve (dF/dT vs temperature) for non-specific peaks. Gene expression was normalized to 18s rRNA. The iCycler iQ Optical System Software version 3.0a (BIO-RAD, Hercules, California) was used to quantify results.

1.2.3. Protein extraction

Protein was isolated from the Trizol layer of eight sheep (four mature and four immature) following the manufacturer's directions (Qiagen Sciences, Germantown, Maryland) during RNA extraction, described above. The pellet was re-suspended in Tissue Protein Extraction Reagent (T-PER; Pierce, Rockford, Illinois) and EDTA-free halt protease inhibitor cocktail, (Pierce, Rockford, Illinois).

1.2.4. SDS-Page and Western Blot

Loading samples were prepared and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using NuPAGE 4-12% Bis-Tris gel in 1X MES SDS running buffer at 110V for one and a half hours. Proteins separated on the gel were then transferred to a polyvinylidene fluoride (PVDF) membrane at 20V for one and a half hours. Membranes were incubated with antibodies directed against Collagen type I (1:500; Abcam, Cambridge, United Kingdom) or Collagen type III (1:500; Abcam, Cambridge, United Kingdom) or Collagen type V (1:350; Millipore, Temecula, CA) of Collagen type VI (1:500; Abcam, Cambridge, United Kingdom) or Decorin (1:500; Santa Cruz Biotecnologies Inc,, Santa Cruz, California) at 4^0C overnight. Membranes were incubated for one hour at room temperature in an HRP-conjugated secondary antibody (1:10,000 Santa Cruz Biotecnologies Inc, Santa Cruz, California). The membranes were developed using an electro-chemiluminescent detection kit (Amersham Biosciences; Buckinghamshire, United Kingdom) and the bundles were quantified

by densitometry. The membrane was stripped and re-probed with antibodies directed against GAPDH (1:500; Invitrogen Corporation, Carlsbad, California) as a loading control. All collagens were analyzed as a ratio to the average of collagen I in each band.

1.2.5. Statistical analysis

ANOVA with Bonferroni post-hoc analysis was used to determine the statistical significance of the real time RT-PCR results. Due to uneven sample size a Kruskal-Wallis test was used to verify the results of the ANOVA with Stata 9 (College Station, Texas). Densitometry measurements of collagen type V, collagen type VI and collagen type III were normalized to the average of collagen type I for each group. Students T-tests and ANOVA with Bonferroni post-hoc was used for the analysis of the protein and fibril diameter data using SPSS (IBM SPSS, Armonk, New York). Significance was considered at $p<0.05$.

1.3. Results

1.3.1. Collagen mRNA expression levels in skeletally mature and immature animals

There were no significant differences between the AM and PL bundles of ACL in any of the collagens (I, III, V, and VI) in both the mature and immature sheep (Figure 1A, B and C). However, examination of the PL bundle of the ACL exhibited significant elevations in the mRNA levels of collagens type I, V and VI in the immature sheep. The mRNA levels of collagens type I, V and VI decreased significantly ($p<0.05$) in the PL bundle of ACL upon maturation (Figure 1A, B and C). The PL bundle exhibited a ~50 fold decrease in collagen I mRNA levels with maturity. Compared to collagen I, collagen V and collagen VI exhibited only a ~9 and ~14 fold decrease respectively in their mRNA levels upon maturation.

A similar pattern of mRNA expression was observed for the AM bundle. The mRNA levels of collagen I, collagen V and collagen VI were significantly elevated ($p<0.05$) in the AM bundle of immature animals when compared to the mature animals. The AM bundle exhibited a ~34 fold decrease in collagen I mRNA levels and collagen V and collagen VI exhibited ~5 and 7 fold decrease respectively upon maturation. Interestingly, collagen III mRNA levels were different in the young and adult sheep from the other collagens discussed above. Collagen III mRNA levels were not significantly different between the young and mature animals for the AM and PL bundles (data not shown).

1.3.2. Assessment of proteoglycan mRNA levels in the AM and PL bundles of ACL

The mRNA levels for small leucine rich proteoglycans, decorin and biglycan, were examined in the AM and the PL bundles. Biglycan mRNA levels were found to be not significantly different ($p>0.05$) for AM and PL bundles of ACL (data not shown). Furthermore, biglycan mRNA levels did not exhibit significant differences in the two bundles between young and mature animals.

The pattern of decorin mRNA levels proved to be different from that of biglycan. The levels of decorin were significantly higher ($p<0.05$) in both the AM and PL bundles of the mature ACLs compared to the juvenile sheep (Figure 2). However, no significant differences were noted in the mRNA levels of decorin between the AM and PL bundles of ACL in both the immature and mature animals. Decorin mRNA levels exhibited a ~5 to 7 fold increase in the PL and AM bundles upon maturation (Figure2)

1.3.3. Changes in matrix proteins with maturation and between bundles of the ACL

All protein proportions measured did not differ significantly between the AM and PL bundles of the ACL. Collagen I and collagen III/average collagen I showed no significant differences between bundles or with maturation (data not shown). However, collagen V/average collagen I and collagen VI/average collagen I levels were significantly higher in immature sheep when compared with mature sheep ($P<0.05$) (Figure 3A, B and C), similar to the mRNA data. No significant differences were observed between the AM and PL bundles of the ACL for both the immature and mature animals in collagens V and VI.

There were no significant differences in decorin protein levels between bundles or with maturation (data not shown).

1.3.4. TEM of collagen fibrils

In normal immature sheep ACLs (AM and PL bundles combined) the mean MFD size (Mean $\pm$ SD: 119.4nm $\pm$ 50.0nm) was significantly smaller than that of the mature sheep (Mean $\pm$ SD: 189.5nm $\pm$ 81.0nm) ACLs ($p <0.001$, Figure 4A). Interestingly there were unique differences between the two bundles of the ACL in the immature sheep. Immature sheep had significantly smaller mean MFD in the PL bundle as compared to the AM band ($p<0.001$ Figure 4B). However, in the mature sheep there was no significant difference between the bundles of the ACL.

In addition to the differences between MFD in juvenile and adult animals, the range and MFD distribution differed. The immature sheep AM bundle's fibril diameters had a range of 283.2nm (max = 310.8nm; min = 27.6nm) while the PL bundle fibril diameters ranged only 124.6mn (max = 153.6nm; min = 29nm). Both bundles in mature sheep had a larger range than the immature sheep, and the range of both the AM band (range = 380.3nm; max = 403.0nm; min = 22.7nm) and PL band (range = 325.3nm; max = 376.0nm; min = 30.7nm) were very similar to one another. There were also distinct differences in the distribution of MFD demonstrating a bimodal distribution of the minimum fibril diameters in the mature sheep and interestingly, in the AM band of the immature sheep. However, the MFD distribution of the PL band of the immature sheep had only a single peak, suggesting more homogeneous fibril sizes (Figure 5). In addition, no differences were observed in fibril outline irregularity (shape or roughening) between the bundles or with maturation.

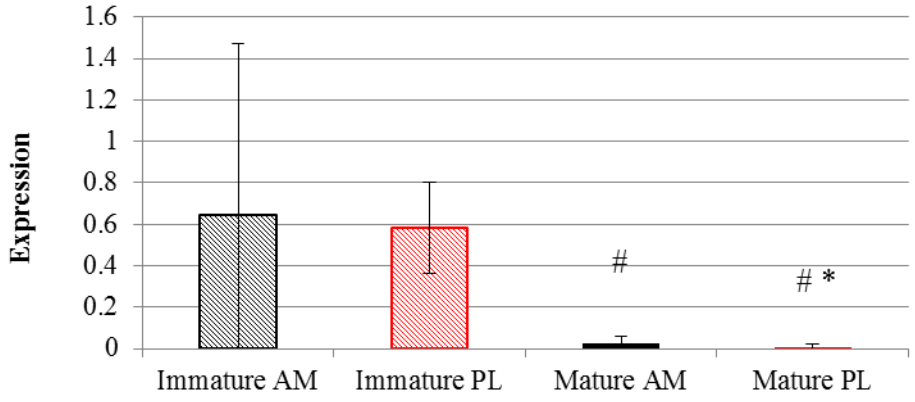
1.4. Figures

1.4.1. Figure 1 Collagen qPCR

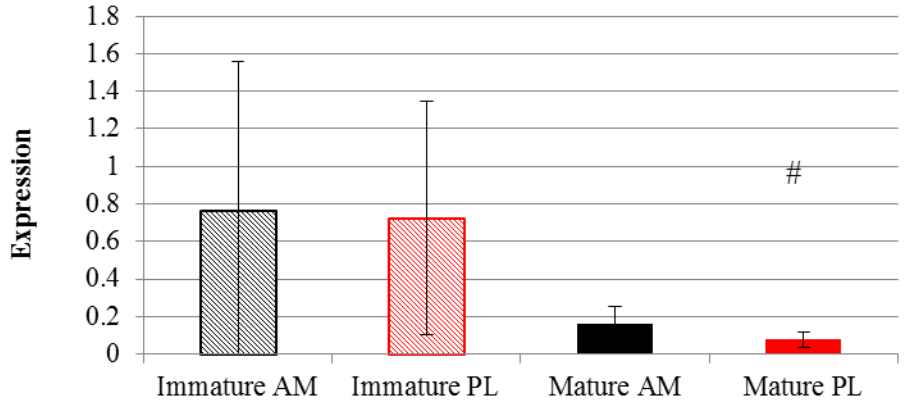
Figure 1: Relative mRNA expression levels of collagens type I (A), type V (B), and type VI (C) in the AM and PL bundles of anterior cruciate ligament (ACL). The immature (1-3 month old) are depicted in hashed boxes and the mature (3-4 years old) are depicted in the solid boxes. Note that individual sample mRNA levels were normalized to 18sRNA before group comparisons. # significantly different from immature AM; $p<0.05$.

* significantly different from immature PL; $p<0.05$.

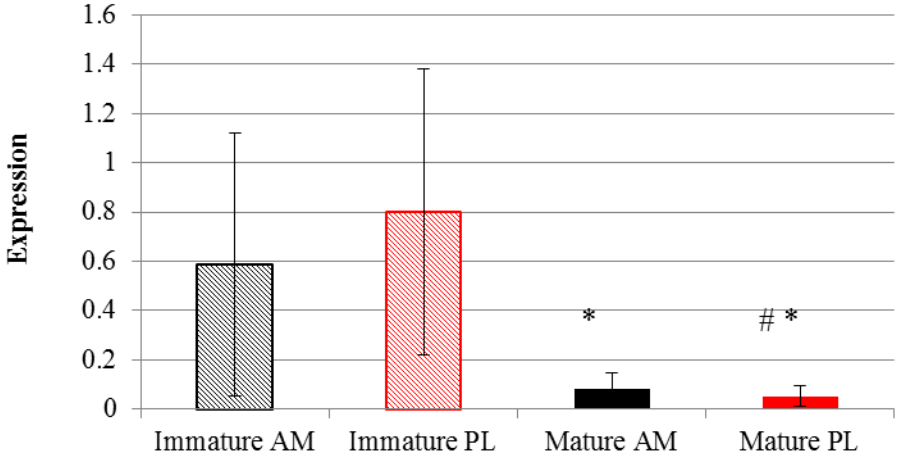
A


Expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
#
*
Immature AM
Immature PL
Mature AM
Mature PL

B


Expression
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
#
Immature AM
Immature PL
Mature AM
Mature PL

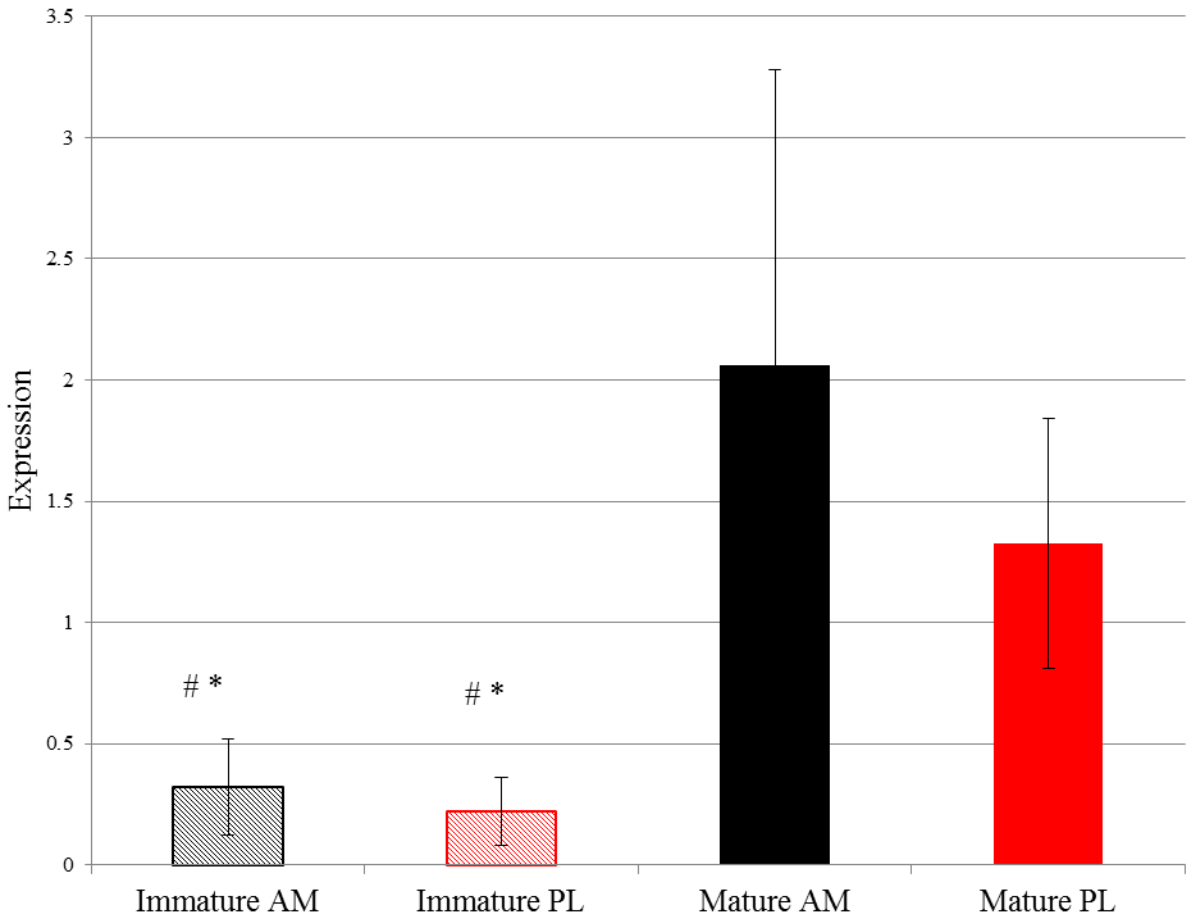
C


Expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Immature AM
Immature PL
Mature AM
Mature PL

1.4.2. Figure 2 Decorin expression

Figure 2: Relative decorin mRNA expression levels in the AM and PL bundles of ACL in the skeletally immature sheep and adult sheep. Note that individual sample mRNA levels were normalized to 18s RNA before group comparisons.

significantly different from mature AM; $p<0.05$. * significantly different from mature PL; $p<0.05$.


3.5
3
2.5
2
1.5
1
0.5
0
Expression
*
*
Immature AM
Immature PL
Mature AM
Mature PL

1.4.3. Figure 3 Collagen protein levels

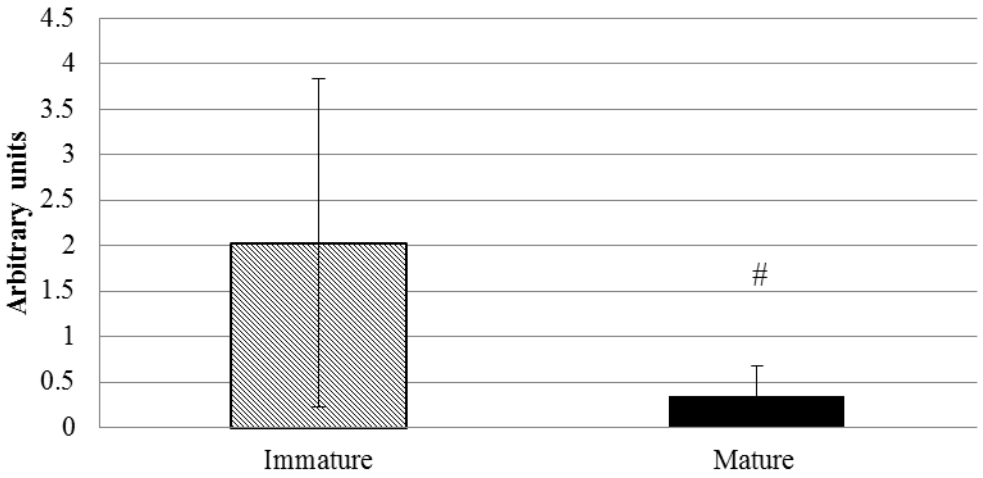
Figure 3: Comparing a ratio of collagen type V/average collagen type I (A) or a ratio of collagen type VI/average collagen type I.

(B) protein levels in the immature (hashed box) and mature sheep (black box) ACL (AM and PL band). The immature sheep ligament has significantly more collagen V than the mature sheep ligament.

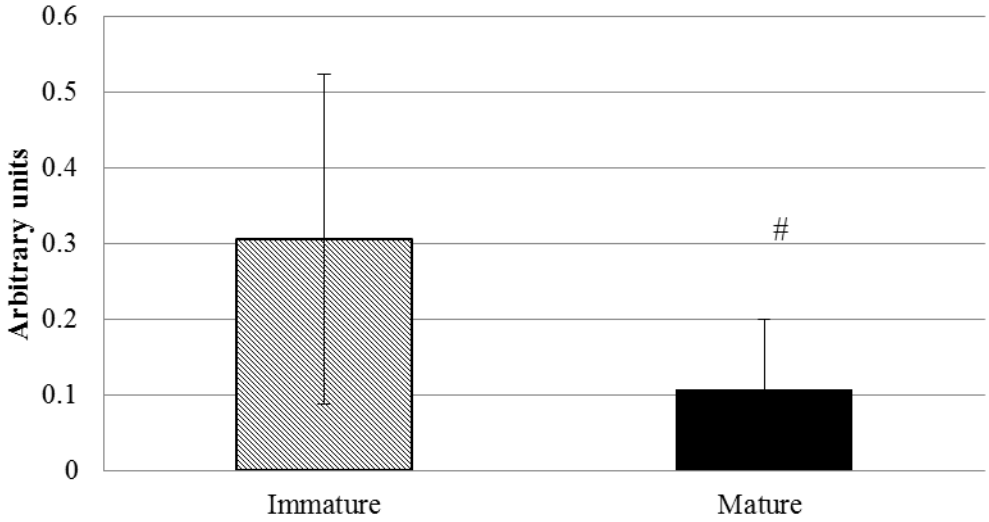
(C) Western Blot depicting the protein levels of collagen type V and collagen type VI in the AM and PL bundles of immature (Lanes 5-9) and mature sheep (1-4).

significantly different from immature; $p<0.05$.

A


4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Arbitrary units
#
Immature
Mature

B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0.4
0.3
0.2
0.1
0
Arbitrary units
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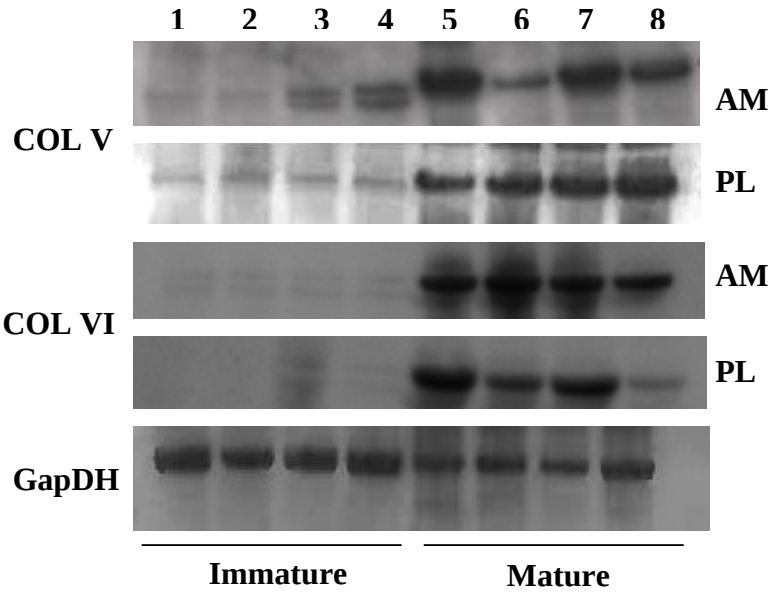
Immature
Mature

C



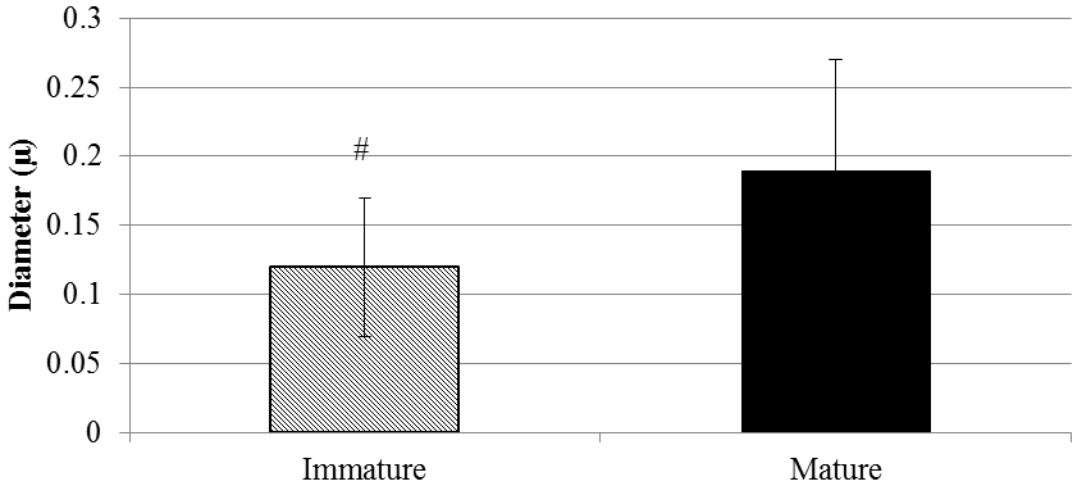
1.4.4. Figure 4 Mean minimum fibril diameter

Figure 4: (A) Comparison of mean minimum fibril diameters of mature sheep (black box) to immature sheep (hashed box). The immature sheep has a significantly lower mean fibril diameter than the mature sheep.

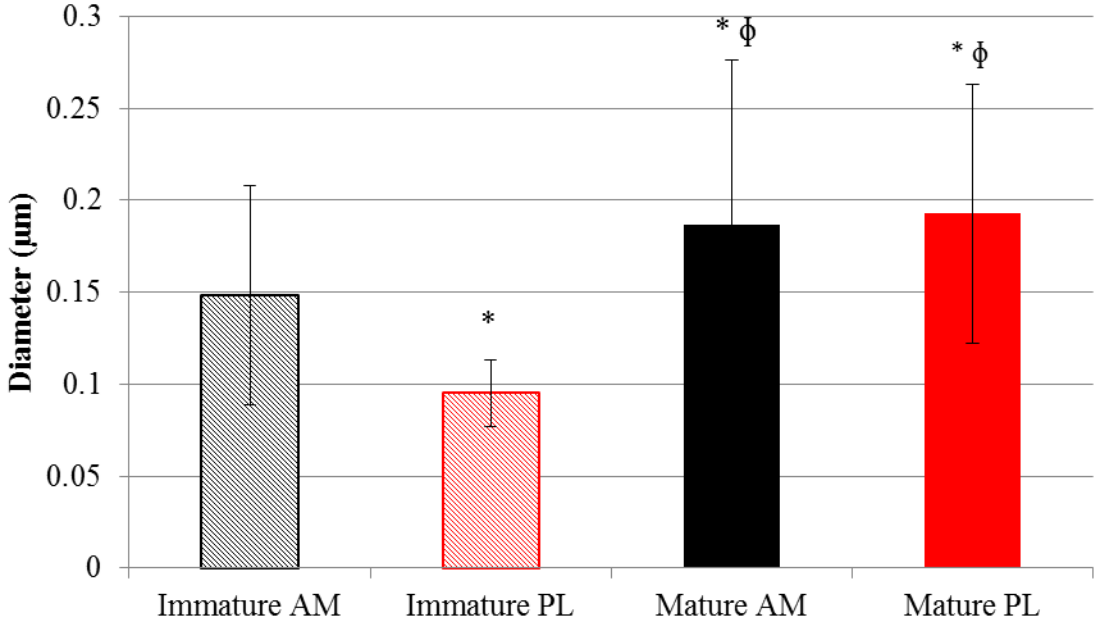
(B) Comparison of bundles of immature and mature sheep. The immature AM band (black hashed box) has significantly larger fibril diameters than the immature PL band (red hashed box).

significantly different from mature; $p<0.001$. * significantly different from immature PL; $p<0.001$. ϕ significantly different from immature AM; $p<0.001$.

A

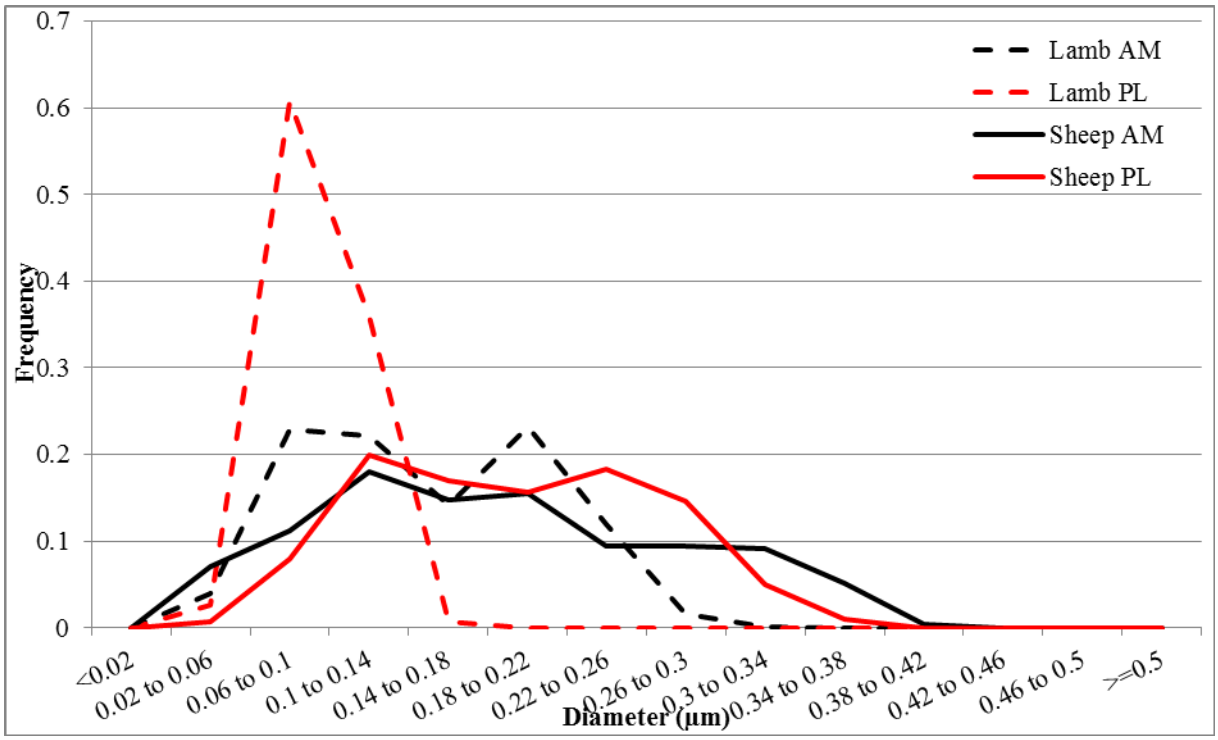

0.3
0.25
0.2
0.15
0.1
0.05
0
Diameter (µ)
#
Immature
Mature

B


0.3
0.25
0.2
0.15
0.1
0.05
0
Diameter (µm)
*
* ϕ
* ϕ
Immature AM
Immature PL
Mature AM
Mature PL

1.4.5. Figure 5 distribution of minimum fibril diameter

Figure 5. A curve depicting minimum fibril diameter distribution. The immature AM band (dashed black line) and both the mature bundles (AM = solid black line; PL = solid red line) have a biomodal fibril distribution. The immature PL band (dashed red line), however, has a unimodal fibril distribution.


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Frequency
Lamb AM
Lamb PL
Sheep AM
Sheep PL
<0.02
0.02 to 0.06
0.06 to 0.1
0.1 to 0.14
0.14 to 0.18
0.18 to 0.22
0.22 to 0.26
0.26 to 0.3
0.3 to 0.34
0.34 to 0.38
0.38 to 0.42
0.42 to 0.46
0.46 to 0.5
>=0.5
Diameter (µm)

1.4. Discussion and Conclusion

The human ACL is made of two bundles: the AM bundle and PL bundle which are thought to be functionally and anatomically distinct (5) and are distinguishable in early foetal development (477). The structural and mechanical properties of ACL have been reviewed in a previous report (476). Although recognised to have functionally distinct roles during flexion and extension of the knee, there are no previous studies which compare the two bundles during skeletal development. In the present study we investigated whether structural and molecular differences exist in the AM and PL bundles of a normal uninjured ACL and whether these differences are maintained over the course of the maturation. This is the first study to date to investigate the structural and molecular differences in the AM and PL bundle of any ACL. Based on the fact that the anatomy of the sheep ACL closely resembles the human ACL, we investigated the structural and compositional changes of the two bundles of ACL during maturation in an ovine model. We hypothesized that due to the different functional roles of the two bundles, they would have distinct matrix composition and ultra-structure that would change during maturation. Our observations of the mature ACL in sheep suggest that it should be considered one ligament with two similar parts that mature at slightly different rates.

Based on our observations of both TEM and collagen levels (collagen type V and collagen type VI) the composition of the immature ACL differs from the adult. With maturation, mean collagen fibril diameter increases, similar to the medial collateral ligament (MCL) and patellar tendon (PT) (494) and collagens type V and VI production decreases. This suggests that while there is some matrix turnover in the adult ACL, the juvenile ACL is likely undergoing more rapid collagen fibrillogenesis. Collagen type V is a quantitatively minor component of type I collagen fibrils found in non-cartilaginous ECM and controls fibril assembly, regulates fibrillogenesis, and plays a critical role in early fibril initiation (495-497). Collagen type VI is

also important in fibril assembly (498), as interconnecting microfibrils (499) which aid in type I collagen fibril arrangement. As both collagen types V and VI are critical for fibril assembly it is not surprising that these collagens were found to be more abundant in immature ACLs, as we found, and that they would be lower with maturation as collagen synthesis decreases (439). This decrease in collagens with maturation corresponds to an increase in MFD in both bundles of the ACL. Once the fibrils have reached maturity they have already been assembled into larger fibrils, previously seen in both the MCL (500, 501) and ACL (501), this may mean that collagen V and collagen VI are no longer as necessary and therefore no longer as abundant.

In the present study we found that both AM and PL bundles of ACL exhibited increased matrix synthesis signals as observed by increased collagen type I, V, and VI mRNA levels in the young and skeletally immature animals when compared to the skeletally mature adult counterparts. Interestingly, there were no significant differences observed in the molecular expression levels between the AM and PL bundles in both the young and adult animals. Although fibrillar collagens have been shown to change with maturation in both the collateral and cruciate ligaments (485), it is interesting to note that AM and PL exhibited similar mRNA levels for these collagens in the young and mature state. Mastrangelo et al. (436) found that ovine cells taken from skeletally immature animals have greater proliferation and migration potential than cells from the adult animal. Increased ACL cell proliferation and migration indicates an increase in cellular activity, which would lead to an increase in collagen I mRNA levels. mRNA levels of collagen types V and VI correlates with an increase in their synthesis as discussed above. This increased matrix building activity has implications for healing potential. Immature healing ACLs have higher cellular density than adult animals (438), which may be beneficial for functional healing of the ligament. In contrast to the expression trends followed by collagen I, collagen V and collagen VI, collagen III mRNA levels exhibited a different pattern.

Collagen III levels remained similar in both the young and the mature animals for the both the AM and the PL band. It has been reported that collagen III forms about 5-18% of total collagen in an ACL graft (502). Several studies have reported that intercalation of minor collagens such as collagen type III and collagen V within the heterotypic actively growing fibrils is responsible for regulating the fibril diameter (503, 504). Alterations in total collagen levels with age have been reported by several groups (439), although in the present study the collagen III mRNA levels did not exhibit any change with maturation. The results for collagen III observed in our study are corroborated by an *in-vitro* study comparing the foetal and adult fibroblasts from ACL tissue (505) where foetal and adult ACL fibroblasts exhibited similar levels of collagen III and collagen type we was found to decrease in adults.. As collagen III up-regulated in repair it was expected that in the present study of normal ACLs, collagen III levels were found to be unchanged with age in contrast to collagen type I which decreased with age.

Despite decreases in collagen expression with age, we found increased decorin expression in mature sheep compared with immature sheep. Very few studies have examined changes in ligament proteoglycan levels during maturation; however, work by Lo et al. (494) demonstrated an increase in decorin expression during rabbit maturation in both the MCL and PT. Decorin is involved in regulating collagen fibril assembly (506), responsible for slowing lateral fusion and increasing fibril resistance to thermal denaturation. Decorin is the most abundant proteoglycan present in ligament extracellular matrix, while biglycan, other large aggregating proteoglycans, versican and aggrecan are less abundant (486, 507). Both decorin and biglycan are thought to play a role in the organization of microfibrillar networks containing collagen type VI, fibrillin or tropoelastin. A study was conducted to compare the collagen types I, II, and III with regard to their ability to bind to decorin and biglycan during fibrillogenesis under *in-vitro* conditions (508). Both decorin and biglycan decreased the proportion of collagen

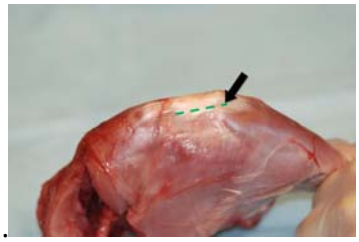
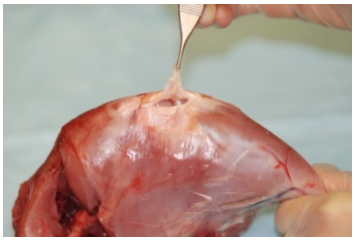
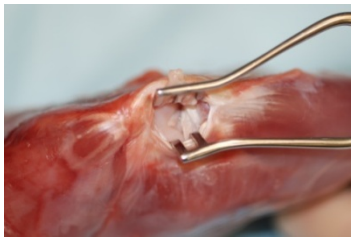
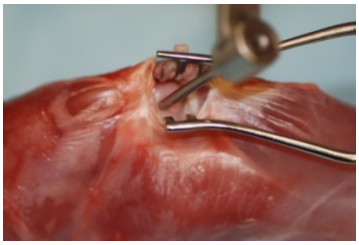
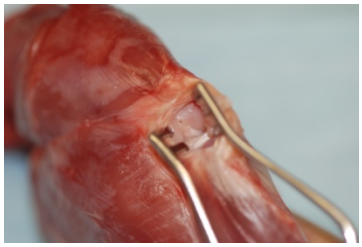
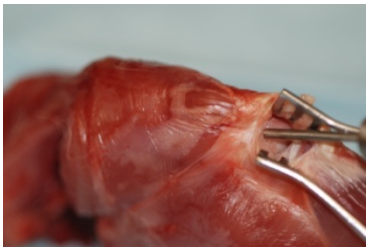
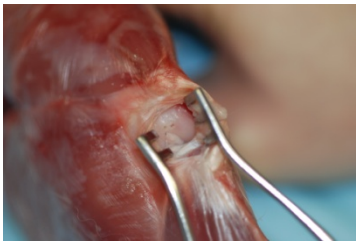
molecules incorporated into fibrils. Interestingly, this study reported that biglycan affected neither fibril diameter nor the speed of fibrillogenesis although decorin reduced the fibril diameter of all three collagen types. In the present study we observe that collagen V was attenuated at maturity although the decorin levels that bind to this collagen were increased. The elevated levels of decorin in the mature ligament can be attributed to its role in maintaining the integrity of the extracellular matrix, and therefore in the biomechanical strength of ligament and further resilience of this tissue to injury and disease.

Rumian et al. (482) found that normal adult sheep ACLs show a bimodal mean fibril diameter distribution, which corroborated earlier work by Parry et al. (509). In the present study, mature adult sheep had a bimodal fibril distribution. Interestingly the MFD of the AM band of the immature animals also had a bimodal distribution, while the MFD of the PL band had a unimodal distribution. Young animals have a unimodal distribution of diameters in a variety of connective tissues; it has been suggested that collagen fibrils may commence growth at different stages of development (509), *i.e.* collagen fibrils reach a limiting diameter creating a unimodal distribution, and then a subset of those fibrils continue to grow at a later phase of development creating a bimodal distribution in adulthood. It is curious that the AM band of the immature ACL shows a bimodal distribution with larger MFD like a mature ligament compared to the smaller MFD and unimodal distribution of the immature PL band. This might suggest that the PL band matures at a slower rate than the AM band of the ACL in sheep. Sakane et al. (510) have demonstrated that the AM bundle has relatively constant levels of in situ forces during knee flexion, whereas the PL bundle has much more variable levels of in situ forces during flexion. In addition to variable in situ forces observed the PL bundle appears to contribute more to rotational stability of the knee during flexion (511, 512). Variations in force distribution may have great importance in ligament healing and how ligaments respond to tensile stress and creep (509).

Therefore, we speculate that slower PL bundle development may be related to its functional roles and may be important for its mechanical properties.

In the ovine model studied here, clear changes were observed in the ACL during maturation. Adult fibrils were larger with lower expression and synthesis of collagen type V and type VI, as well as increased decorin expression. The immature ACL is more active and actively building matrix, this has critical implications in the healing potential of the ACL following injury in skeletally immature individuals, and possible interventions for increasing healing in a mature ligament. Interestingly, while the AM and PL band are molecularly and structurally similar in the adult sheep, the ultra-structure differed in the juvenile sheep demonstrating that the PL band matures more slowly than the AM band of the ACL. This study also determined that the adult ACL should be considered as one bipartite structure as opposed to two unique structures (AM and PL bundles). These observations have implications on functional assessment of the bundles, as well as their surgical reconstruction. Although the AM and PL bundles are similar at the molecular levels in the normal uninjured ACL, it remains to be determined whether they exhibit differences at molecular levels after ligament injury as this may explain the poor healing potential of this single bipartite ligament.

2. SURGICAL APPROACH FOR RABBIT MODEL OF PTOA

1. 
2. 
3. 
4. 
5. 
6. 
7. 

1. Approach via the lateral aspect of the knee. Make an incision just below the patellar tendon the length of the joint. Incision line indicate by dashed line and arrow.
2. Reflect back the fat pad from underneath the patella.
3. Retract the tissue from around the joint in order to get a good line of sight
4. Position drill guide in the femoral notch (no articular cartilage).
5. Drill a 0.5mm wide 10mm deep hole with a Dremel.
6. Reposition the drill guide to a new site just beside the original hole.
7. Drill a second hole into the femoral notch same depth and diameter as above.

3. SUPPLEMENTAL TABLES

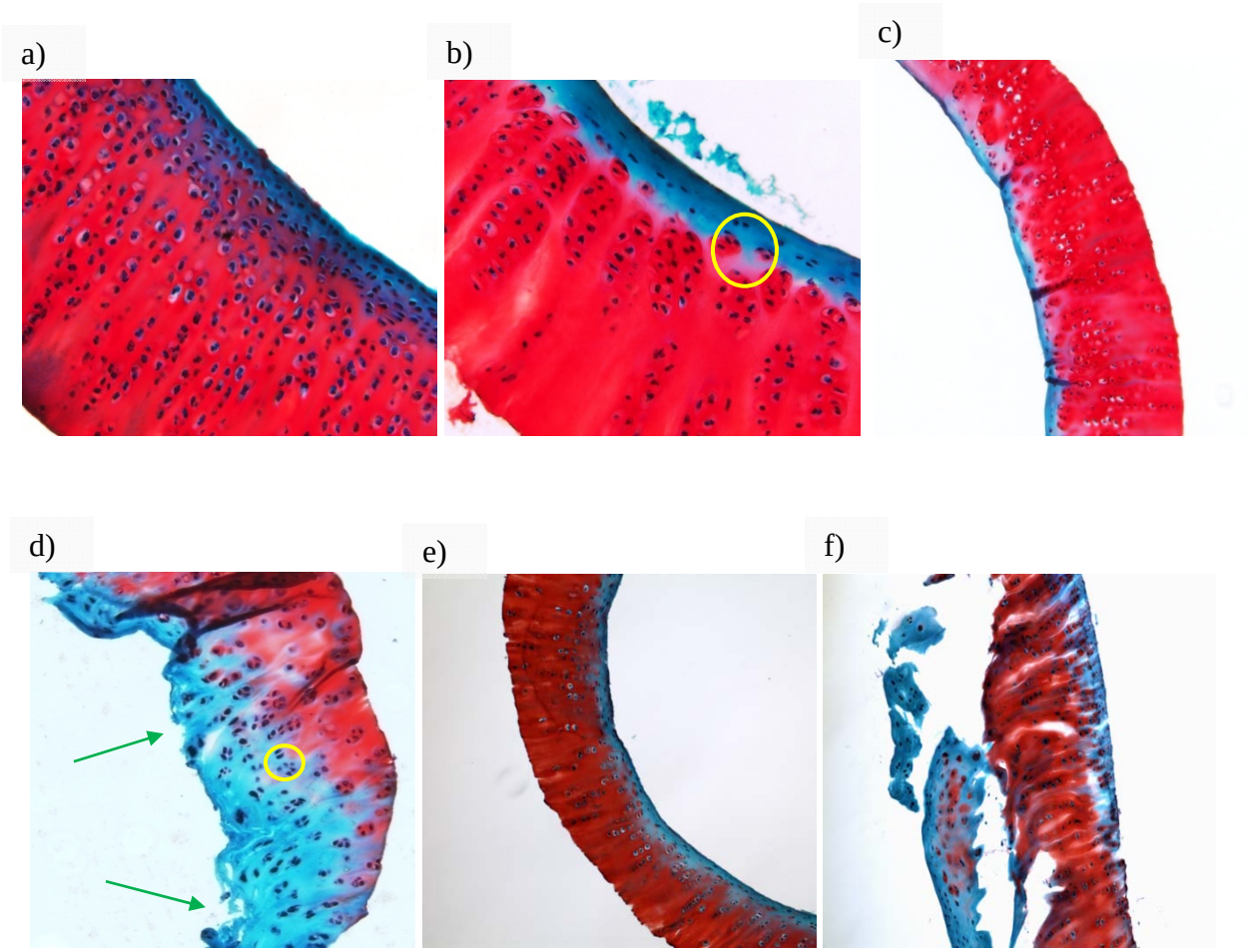
3.1. Table 1. Modified Mankin Score

Table 1. Modified Mankin Score		
Histological parameter		**Score**
I. Structure		
(a)	normal	0
(b)	slight surface irregularities	1
(c)	moderate surface irregularies	2
(d)	severe surface irregularies	3
(e)	clefts into the transitional zone (1/3 depth)	4
(f)	clefts into the radial zone (2/3 depth)	5
(g)	clefts into the calcified zone (full depth)	6
(h)	fibrillation +/or loss to transitional zone (1/3 depth)	7
(i)	fibrillation +/or loss to radial zone (2/3 depth)	8
(j)	fibrillation +/or loss to calcified zone (full depth)	9
II. Cellularity		
(a)	normal	0
(b)	increase or slight decrease	1
(c)	moderate decrease	2
(d)	severe decrease	3
(e)	no cells	4
III. Cell Cloning		
(a)	normal	0
(b)	several doublets	1
(c)	many doublets	2
(d)	doublets and triplets	3
(e)	multiple nests	4
IV. Safranin-O		
(a)	normal	0
(b)	slight reduction	1
(c)	staining reduced in radial layer	2
(d)	reduced in interterritorial matrix	3
(e)	only present in pericellular matrix	4
(f)	no staining	5

3.2. Table 2. qPCR Primers

Table 2. Primer sequences used for qPCR analysis			
Gene		Primer Sequence	Gene Bank Accession No.
18s	F	5'-TGG TCG CTC GCT CCT CTC C-3'	X 03205
	R	5'-CGC CTG CTG CCT TCC TTG G-3'	
RABBIT			
MMP13	F	5'-TTC GGC TTA GAG GTG ACA GG-3'	AF 059201
	R	5'-ACT CTT GCC GGT GTA GGT GT-3'	
MMP3	F	5'-GCC AAG AGA TGC TGT TGA TA-3'	M 25664
	R	5'-AGG TCT GTG AAG GCG TTG TA-3'	
TGFβ	F	5'-CGG CAG CTG TAC ATT GAC TT-3'	AF 000133
	R	5'-AGC GCA CGA TCA TGT TGG AC-3'	
IL1β	F	5'-TAC AAC AAG AGC TTC CGG CA-3'	D 23815
	R	5'-GGC CAC AGG TAT CTT GTC GT-3'	
IL1Ra	F	5'-AAG GAG CAG GAC AAG CGC TTC A-3'	NM 173842
	R	5'-CAG GAG GAC AGC AGA GGT G-3'	
IL6	F	5'-CCT GCC TGC TGA GAA TCA CTT-3'	AF 169176
	R	5'-CGA GAT ACA TCC GGA ACT CCA T-3'	
IL8	F	5'-CAA CCT TCC TGC TGT CTC TG-3'	AF 099665
	R	5'-GGT CCA CTC TCA ATC ACT CT-3'	
Col I	F	5'-GAT GCG TTC CAG TTC GAG TA-3'	Hart D, Reno CR
	R	5'-GGT CTT CCG GTG GTC TTG TA-3'	personal communication
bFGF	F	5'-TAC AAC TTC AAG CAG AAG AG-3'	X 04432, X 04433
	R	5'-CAG CTC TTA GCA GAC ATT GG-3'	
MCP1	F	5'-CTT CTG TGC CTG CTG CTC ATA G-3'	X 109720
	R	5'-TGC TTG GGG TCA GCA CTGA T-3'	
TNFα	F	5'-TCT AGT CAA CCC TGT GGC CC-3'	M 12845
	R	5'-GCC CGA GAA GCT GAT CTA AG-3'	
SHEEP			
Biglycan	F	5'-TCG CTG CTC TCA GAC GAC AC-3'	AI 931862
	R	5'-CAT TAT TCT GCA GGT CCA GC-3'	
Decorin	F	5'-CAA ACT CTT TTG CTT GCG CTG-3'	AF 125041
	R	5'-CAC TGG ACC ACT CGG AGA TG-3'	
Col I	F	5'-CAC AAG GAG TCT GCA TGT CT-3'	AY 091602
	R	5'-GTT CAC CAG GCT CAC CAG CA-3'	
Col III	F	5'-GCT GGC TAC TTC TCG CTC TG-3'	AY 091605
	R	5'-GTG GGc AAA CTG CAC AAC AT-3'	
Col V	F	5'-GCA GAC TCT GTG GAT GTG CT-3'	AB 097499
	R	5'-CTG CCA TTC ACA CTG ACT GC-3'	
Col VI	R	5'-ATC GAC TCG GAG GAG GTT GG-3'	AF 129288
	F	5'-CTT CAC GTT GTC CGA CGA GG-3'	

4. REPRESENTATIVE HISTOLOGICAL SECTIONS WITH SAFRANIN-O/FAST GREEN STAIN



Micrographs of 5µm histological sections of cartilage stained with Safranin-O/Fast Green. a,c,e) are of Ctl cartilage (20x and 10x). b) a representative micrograph of 6 week Drill animal (20x); there is clear chondrocyte cloning (yellow circle). d) a representative micrograph of 9 week Drill, in which there is a loss of safranin-O staining, marked surface damage (green arrows; 10x), and cell cloning (yellow circle; 10x). f) a representative section of 1 year Drill cartilage; the cartilage has lost all of its integrity with large fissures and clefts throughout (10x).

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