Investigating the role of interleukin-15 in post-traumatic osteoarthritis

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Physiology and Pharmacology
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Abstract

Post-traumatic Osteoarthritis (PTOA) is a degenerative joint disease, leading to articular cartilage breakdown, osteophytes, and synovitis, caused by an initial joint trauma. Pro-inflammatory cytokines increase catabolic activity and may perpetuate inflammation following joint trauma. Interleukin-15 (IL-15), a pro-inflammatory cytokine, is increased in OA patients, although its exact role in the disease pathology is unknown. Using \textit{Il15} deficient rats, this study investigated the role of IL-15 in PTOA pathogenesis in an injury-induced model of OA. Semi-quantitative scoring of the articular cartilage, subchondral bone, and osteophyte formation reveals no significant difference between \textit{Il15} deficient rats and wild-type rats, following PTOA-induction. Similarly, synovitis scoring across 6 parameters found no significant difference between genetic variants. Overall, IL-15 does not appear to play a key role in PTOA pathogenesis in this model.

\textbf{Keywords:} Interleukin-15, post-traumatic osteoarthritis, articular cartilage, synovial joint, transgenic rats
Summary for Lay Audience

Post-traumatic Osteoarthritis (PTOA) is a degenerative joint disease, leading to cartilage breakdown and changes to the surrounding tissue, caused by an initial joint injury. Pro-inflammatory cytokines may increase joint damage and cause chronic inflammation following joint trauma. Interleukin-15 (IL-15), a pro-inflammatory cytokine, is increased in OA patients, although its exact role in the disease is unknown. Using Il15 deficient rats, this study investigated the role of IL-15 in PTOA progression in an injury-induced model of OA. The cartilage, bone, and joint capsule were analyzed to assess PTOA damage. Examination of the joint tissue reveals no significant difference between Il15 deficient rats and control rats, following PTOA-induction. Overall, IL-15 does not appear to play a key role in PTOA progression in this model.
Co-Authorship Statement

All data presented in this thesis was collected and analyzed by Ermina Hadzic. Dr. Stephen Renaud provided the initial $\text{Il15}^{-/-}$ rats, which were bred in house and maintained by Ermina Hadzic. Garth Blacker performed all of the anterior cruciate ligament and destabilized medial meniscus (ACLT-DMM) surgeries to induce OA and assisted in semi-quantitative scoring. Holly Dupuis assisted in tissue collection and semi-quantitative scoring. Dr. Frank Beier contributed to the study design and editing of the thesis.
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ACL</td>
<td>Anterior Cruciate Ligament</td>
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<tr>
<td>ACLT-DMM</td>
<td>Anterior Cruciate Ligament Transection with Destabilized Medial Meniscus</td>
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<tr>
<td>ADAMTS</td>
<td>A Disintegrin and Metalloproteinase with Thrombospondin-like Motifs</td>
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<tr>
<td>BMP</td>
<td>Bone Morphogenic Protein</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<tr>
<td>DAMP</td>
<td>Damage Associated Molecular Patterns</td>
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<tr>
<td>DMOAD</td>
<td>Disease Modifying Osteoarthritis Drug</td>
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<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
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<tr>
<td>FLS</td>
<td>Fibrous-like Synoviocytes</td>
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<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
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<tr>
<td>IACS</td>
<td>Intra-Articular Corticosteroid</td>
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<td>IAHA</td>
<td>Intra-Articular Hyaluronan</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>KL</td>
<td>Kellgren-Lawrence</td>
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<tr>
<td>LFC</td>
<td>Lateral Femoral Condyle</td>
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<td>LTP</td>
<td>Lateral Tibial Plateau</td>
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<tr>
<td>MAC</td>
<td>Membrane Attack Complex</td>
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<tr>
<td>MFC</td>
<td>Medial Femoral Condyle</td>
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<tr>
<td>MLS</td>
<td>Macrophage-like Synoviocytes</td>
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<tr>
<td>MMP</td>
<td>Matrix Metalloproteinases</td>
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<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
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<tr>
<td>MTP</td>
<td>Medial Tibial Plateau</td>
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<tr>
<td>NGF</td>
<td>Nerve Growth Factor</td>
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<tr>
<td>NSAID</td>
<td>Non-Steroidal Anti-Inflammatory Drug</td>
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<tr>
<td>OA</td>
<td>Osteoarthritis</td>
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<td>OCT</td>
<td>Optical Coherence Tomography</td>
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<tr>
<td>PCL</td>
<td>Posterior Cruciate Ligament</td>
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<td>PTOA</td>
<td>Post-Traumatic Osteoarthritis</td>
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<tr>
<td>RA</td>
<td>Rheumatoid Arthritis</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>ROM</td>
<td>Range of Motion</td>
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<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
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<tr>
<td>STZ</td>
<td>Superficial/Tangential Zone</td>
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<tr>
<td>TGF</td>
<td>Tumour Growth Factor</td>
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<tr>
<td>TKR</td>
<td>Total Knee Replacement</td>
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<tr>
<td>TLR</td>
<td>Toll-Like Receptor</td>
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<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
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<tr>
<td>PRR</td>
<td>Pattern Recognizing Receptor</td>
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<td>ZFN</td>
<td>Zinc Finger Nucleases</td>
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Chapter 1:
Introduction
1 Introduction

Musculoskeletal conditions are the leading cause of disability in Canada, with Osteoarthritis (OA) as the second most prevalent condition after lower back pain [1]. Arthritis is the leading chronic condition for Canadian adults and is expected to rise to almost 9 million cases by 2040 [2]. OA is a degenerative joint disease that leads to cartilage breakdown, boney deformation, and synovial inflammation [3]. Those impacted may have one or more synovial joints affected, with the hip, knee, hand, and spine most common, leading to limited mobility and pain [4]. OA pain results in people with arthritis being much more likely to report disability within all age groups and to drop out of the labour force [2]. The total direct cost of OA in Canada is expected to increase 2.6-fold by 2031, to $7.6 billion dollars, demonstrating its great economic burden [5]. Currently, there is no disease modifying agent for OA (DMOAD), so treatment focuses on pain management and total joint replacement in the end stage. In this chapter, I will examine the synovial knee joint, its response to OA, and the role of inflammatory mediators.

1.1 The Synovial Joint

Joints that have a synovial cavity between two articulating bones are classified as synovial joints. They have a range of motion depending on the shape of the articulating bones. Joints are complex organs, comprised of many different tissues that function to provide structural support, lubrication, and shock absorption. Synovial joints consist of a joint capsule (separated into a fibrous and synovial membrane), articular cartilage, subchondral bone, synovial fluid, and supporting structures (Fig 1.1) [6]. This section will further explore each structure within the joint, in order to understand their role in OA pathophysiology.
Figure 1.1 – The synovial joint in a healthy and osteoarthritic knee

The synovial joint is a dynamic organ, comprised of a joint capsule, articulating cartilage, subchondral bone, synovial fluid, and supporting structures. These tissues all work together in order to provide structural support, lubrication, and shock absorption. Osteoarthritis affects each joint tissue in a unique way, resulting in articular cartilage breakdown, synovitis, and altered subchondral bone.
1.1.1 Articular Cartilage

Cartilage is a prominent structural tissue within the body, distinguished into three categories: hyaline, elastic, and fibrous. Hyaline cartilage is the most abundant type in the body and plays an integral role within the joint. Articular cartilage is composed of hyaline cartilage, lining the ends of bones in diarthrodial joints. It functions to decrease friction and absorb/distribute forces during movement. The hyaline cartilage within the metaphysis has a specific role in long bone growth [7]. Cartilage is composed of one cell type, termed chondrocytes, that accounts for only 5% of the total tissue volume in humans. Chondrocytes are derived from mesenchymal progenitor cells but show little to no proliferation in adult tissue. The remaining 95% of cartilage is the extracellular matrix (ECM), with a vast majority (70-80%) of the ECM being water, with collagens and proteoglycans comprising the remainder. The tissue is avascular, depending on the surrounding synovial fluid for nutrient diffusion. The ECM collagen is overwhelmingly (90%) type II, with type I collagen only found in small amounts at the superficial zone [8]. The remaining 10% is believed to include type VI, IX, X, and XI collagens, although minor contributions of additional collagens have been described [8, 9]. The collagens provide structural support, as well as tensile and shear resistance. Additionally, the collagen helps restrain the proteoglycans in the ECM. Amongst the ECM proteoglycans, aggrecan is the most abundant, consisting of a core protein and glycosaminoglycan (GAG) side chains of chondroitin sulphate and keratin sulphate. The aggrecan is non-covalently linked to a hyaluronic acid backbone through Link protein, creating a large polymer that is easier for the collagen to restrain. Most important is the negative charge of GAGs, as it is this feature that allows the proteoglycans to attract water during compression [8].

Articular cartilage is classified into zones based on chondrocyte and collagen organization (Fig 1.2). The zones are as follows: (1) Superficial/Tangential Zone (STZ), (2) Middle/Transitional Zone, (3) Deep/Radial Zone, and (4) Calcified Zone [8]. The STZ comprises the upper 10-20% of tissue, with flat discoidal chondrocytes and densely packed collagen fibres oriented parallel to the surface. This zone is adapted to reduce
friction through the secretion of specialized proteins, a high water content, and parallel collagen/chondrocyte arrangement. In fact, this zone demonstrates a 2 times greater Young’s modulus compared to the Middle and Deep Zones. The next 40-60% is the Middle Zone, with round chondrocytes in perpendicular columns and randomly oriented collagen fibres. This zone has the highest proteoglycan content. The Deep Zone comprises the last 30-40%, where the chondrocytes beginning to become more spherical and stack perpendicularly. The collagen fibres are thicker and serve to anchor the cartilage to the subchondral bone via perpendicular bundles [8]. Finally, the Tidemark is the distinguishing line between the calcified and non-calcified cartilage, establishing a transition between the cartilage and subchondral bone. The tidemark is part of the osteochondral junction, a functional unit consisting of the deep non-calcified cartilage, tidemark, calcified cartilage, cement line, and subchondral bone. This area has a strong biomechanical and biochemical connection, adapting to any changes occurring to the tissues [10].
Articular cartilage is organized into four distinct zones based on the organization and characteristics of chondrocytes and collagen fibres. The Superficial Tangential Zone (STZ) has flattened chondrocytes with collagen fibres oriented parallel to the cartilage surface and is adapted to reduce friction. Next, the Middle Zone has round chondrocytes with collagen fibres in a random orientation, containing the highest proteoglycan content. In the Deep Zone, the chondrocytes and collagen fibres are perpendicularly stacked. Here, the collagen fibres are thicker and serve to anchor the non-calcified cartilage to the subchondral bone. Finally, the calcified cartilage is separated from the non-calcified cartilage by the tidemark. The tidemark, cement line, and subchondral bone are all part of the osteochondral junction, a highly responsive area.

**Figure 1.2 – Articular cartilage is organized in distinct zones**
1.1.2 Bone & Subchondral Bone

Bones are the structural framework of the body, accounting for 18% of body weight [11]. Additional functions include organ protection, load bearing, generating movement with coordinating muscles, blood cell production, endocrine functions, and mineral and triglyceride storage. Multiple tissue types make up bones, such as marrow, periosteum, endosteum, as well as a nerve and blood supply. Bone is majorly comprised of the mineral Calcium Phosphate ($Ca_3(PO_4)_2$), as well as an organic component of type I collagen and non-collagenous proteins. The collagen fibres are layered at varying angles in order to provide greater structural integrity, guiding the direction of $Ca_3(PO_4)_2$ organization. Bone is subdivided into two categories: cortical/compact bone making up the majority of the diaphysis, and the trabecular/spongy bone at the core of the epiphysis. Three functional cell types contribute to bone tissue: osteoblasts, osteoclasts, and osteocytes. Osteogenic cells are derived from mesenchymal stem cells and differentiate into osteoblasts, which secrete collagen and other proteins in order to build bone ECM, eventually becoming trapped and maturing into osteocytes. Alternatively, osteoblasts may also become inactive bone lining cells or go through apoptosis. The osteocytes are found in lacunae, which are connected via small channels called canaliculi. Within these channels, neighboring osteocyte processes create gap junctions in order to communicate and maintain metabolism. Finally, osteoclasts, derived from myeloid lineage, are bone-resorbing cells, utilizing secretions of lysosomal enzymes and acid from its ruffled border to breakdown collagen and $Ca_3(PO_4)_2$. The balance of osteoblast formation and osteoclast resorption defines the bone remodeling cycle, an asynchronous process integral to bone growth, maintenance, and repair [11].

The definition of subchondral bone is not universal, varying from the tissue immediately below the articular cartilage tidemark, to being the trabecular bone adjacent to the cortical bone [12]. That being said, it is widely accepted that there are two distinct anatomical entities within the subchondral bone - the bone plate and the supporting trabeculae. The subchondral bone plate is the cortical endplate immediately under the calcified cartilage, or cement line. The subchondral trabecular bone, also referred to as
supporting trabeculae, is the area under the bone plate consisting of the trabecular bone and deeper bone structures [10, 12]. For the purpose of this thesis, “subchondral bone” refers to the subchondral bone plate and the supporting trabeculae. The subchondral bone plate is porous, with channels containing blood vessels and nerves from the marrow penetrating into the calcified cartilage. Interestingly, these dynamic channels vary with joint stress and cortical thickness. More channels will form in areas of high joint stress, such as the center of the tibial plateau, with a 15-25% increase in vessel density in these areas. As for cortical thickness, the channels are more narrow in thicker regions, leading to branch-like projections, compared to the wide, ampullae-like channels in the thinner areas [10, 12]. The bone plate varies in part due to the varying shape of the articulating surface, where the periphery is thinner. A regular and concentric thickness distribution is commonly found, for example the tibial plateau has a 7- to 12-fold increase from the periphery to the center [12]. Underneath, the supporting trabeculae play an important role in shock absorption and metabolism. The tissue has an increased porosity compared to the cortical bone, containing blood vessels, nerves, and bone marrow, allowing for high metabolic activity. Additionally, its structure varies depending on articular cartilage distance [10].

Subchondral bone plays a vital role in load bearing, working in complement with the articular cartilage. The bone absorbs the mechanical load from the articular cartilage, gradually transitioning the stress/strain [10]. The composition of bone is specialized to absorb this load from the overlying cartilage, as it is much stiffer and stronger. This is believed to aid in the bones ability to additionally dissipate juxta-articular loads [12]. Overall, the subchondral bone is a dynamic tissue, playing a major role in joint health and metabolism.

1.1.3 Synovium

The synovium is a vital joint structure, maintaining homeostasis, providing nutrients, and clearing waste. It is comprised of the synovial membrane, or intima, (mainly synoviocytes), and the sub-intimal space (fibrous connective tissue and blood vessels).
The synovium’s secretion of synovial fluid is one of its most important functions. This viscous fluid functions to reduce friction, aid in shock absorption, supply oxygen/nutrients, and remove waste. Without it, the avascular cartilage would not be able to maintain homeostasis [6]. Synovial fluid is derived from interstitial fluid as a blood ultra-filtrate, containing hyaluronic acid and lubricating molecules, which provide non-Newtonian properties to the fluid. Additionally, there are inorganic salts such as sodium, potassium, calcium, and chloride within the fluid. When shear force is applied, the fluid viscosity increases in order to absorb the force, and then immediately decreases viscosity with either the removal of force or prolonged stress [8]. There are two types of synoviocytes within the synovial membrane: the fibrous-like synoviocytes (FLS) are specialized secretory cells, and the macrophage-like synoviocytes (MLS) clear joint debris [13]. The FLS (type B synoviocytes) are the predominant cell type, accounting for 75% of all cells in the intima. Collagen, fibronectin, hyaluronic acid, lubricin, and other GAGs are all secreted by the FLS into the synovial fluid, under the influence of signals from the neighboring fenestrated capillaries [13, 14]. Hyaluronic acid plays an important aspect in joint health, maintaining synovial fluid viscosity and assisting in joint cushioning, while lubricin is the most prominent lubricating protein [13, 15]. Finally, in vitro and ex vivo studies have found the potential for FLS to differentiate into chondrocytes, providing a cell source for cartilage regeneration [13].

The MLS (type A synoviocytes) play a key role in joint health as they are specialized to phagocytose ECM constituents, cell debris, microorganisms, and antigens in the synovial fluid [14]. They also regulate the levels of pro- and anti-inflammatory cytokines through their phenotypical variants. M1, or classically activated macrophages, are pro-inflammatory, while the M2, or alternatively activated macrophages, are anti-inflammatory mediators. Following joint trauma, bone marrow derived macrophages (non-tissue resident) are quickly recruited due to their proximity to blood vessels, differentiating into M1 or M2 after infiltrating the synovium in order to regulate healing [16].
The sub-intima is categorized as fibrous, areolar, or adipose tissue, containing type I collagen and a healthy supply of blood, nerve, and lymphatic vessels. This layer is up to 5 mm thick, although there may be no discrete membrane in some areas due to surrounding tissue. The areolar type is the most specialized type of sub-intima, with viscoelastic properties to cope with stretching and rolling during dynamic movement [17].

1.1.4 Supporting Structures

Supporting structures play an additional role in joint stability and mobility, varying across joints based on their required function. These include, but are not limited to, ligaments, tendons, menisci, bursa, and labrum [6]. Ligaments may be categorized as extra- or intra-capsular, an important distinction both anatomically and in terms of swelling. In the knee, the collateral ligaments are extra-capsular and the cruciate ligaments are intra-capsular, the latter of which has significantly increased swelling following injury. Regardless, both types play an important role in joint stability during dynamic movement [18, 19]. The knee also contains two menisci, a fibrocartilage structure resembling a disc that assists in shock absorption [6]. The menisci are stabilized within the joint via the transverse ligament, as well as attachments to the femur and tibia to prevent dislodging during compression. Blood supply is limited to the outer portion of the tissue, leaving the remaining 65-75% depending on the synovial fluid for nutrients. The meniscus has functions in addition to shock absorption, such as secondary joint stabilization, stress distribution, joint protection, and cartilage nutrition and lubrication. Meniscus loading across the tissue varies from 45-70% of the weight-bearing load, demonstrating a significant role in attenuating forces to the cartilage and bone. Similar to the articular cartilage, the collagen fibres (type II here) demonstrate zonal organization in order to best distribute load [20]. Finally, the muscles and tendons work together with the bone to produce movement and further stabilize the joint [6].
1.2 OA Pathophysiology

OA is a disorder of the entire joint, initially categorized by abnormal joint tissue metabolism and later by physiological changes, including cartilage degeneration, bone remodeling, osteophyte formation, inflammation, and loss of joint function (Fig 1) [3]. It is more than just a disorder of the articular cartilage, with varying effects on each tissue in the joint. Each tissue reacts in its own way to the disease, as well as to the actions of the other joint tissues. OA is a heterogenous, multifactorial disorder, leading to varying presentations of the disease. This section serves as an overview of the effects of OA on each joint tissue, with a closer analysis of variation in Section 1.6.

1.2.1 Articular Cartilage Effects

OA can be initiated through a number of mechanisms, including injury. The articular cartilage is generally the first tissue thought of when discussing the effects of OA. Early in the disease, chondrocytes will attempt to proliferate to repair damage, which results in chondrocyte clusters as well as an increase in ECM secretion. Subsequently, catabolic activity increases in the joint, leading to the degradation of type II collagen and the proteoglycans within the ECM. Additionally, changes to regulatory proteins, matrix proteins, stress markers, apoptotic markers, and transcription factors are found [21]. Matrix metalloproteinases (MMPs) are zinc-dependent enzymes that play a major role in ECM degradation through the cleaving of type II collagen. Additionally, they may cleave cell surface receptors and other ECM components (e.g., aggrecan, fibronectin, laminin). MMPs are synthesized and secreted by fibroblasts and chondrocytes in the joint, influenced by the activity of cytokines. Many MMPs have been studied within OA pathophysiology, with MMP-1, -8, and -13 as the major collagenases. Increased MMP-13 expression is one hallmark of OA and has a preference for destruction of type II collagen [22]. Interestingly, MMP-13 is secreted by chondrocytes in healthy adults, but is quickly endocytosed and destroyed [23]. Animal studies have been instrumental in the understanding of the role of MMP-13 and OA. Neuhold et al. (2001), found that mice
with a cartilage-specific increase in MMP-13 activity had increased articular cartilage destruction, similar to what is seen in OA pathology [24]. Further work by Little et al. (2009) used an MMP-13<sup>-/-</sup> mouse model with surgically induced OA and found that the MMP-13<sup>-/-</sup> mice had less cartilage damage, compared to wild-type mice [25]. This was confirmed by Wang et al. (2013), who used a mouse model with cartilage-specific MMP-13 deletion, finding a decrease in cartilage damage at 8, 12, and 16 weeks post OA-inducing surgery [26]. Overall, the MMPs, and specifically MMP-13, work to decrease the structural integrity of cartilage through cleavage of type II collagen and other substrates [22 – 26].

Cartilage integrity is diminished further by ADAMTS aggrecanases (a disintegrin and metalloproteinase with thrombospondin-like motifs), which breakdown the ECM. In fact, aggrecan loss is the first measurable difference in the ECM during OA pathogenesis [27]. The loss of aggrecan is primarily due to ADAMTS aggrecanases, with ADAMTS-4 and -5 studied the most with respect to OA. Both work to cleave aggrecan at the aggrecanase-specific Glu373-Ala374 site and are the most efficient aggrecanases [27]. A 2002 study by Malfait et al. determined that inhibition of ADAMTS-4 and -5 (via BB-16) successfully blocked aggrecan destruction in OA cartilage in vitro [28]. Majamdar et al. (2007) later generated ADAMTS-4/5 double knockout mice and surgically induced OA to further study their role. They found that the double knockout mice were protected against OA-related proteoglycan loss, which was comparable to the ADAMTS-5 single knockout mice, but not the ADAMTS-4 single knockout mice [29]. That being said, ADAMTS-5 has not been widely accepted as the main aggrecanase in humans as the relationship is more complex [27]. The loss of aggrecans in the ECM results in cartilage that is not able to hold in water, thus losing its viscoelastic properties.

Damage to the articular cartilage can be tracked histologically in animal models, where surface fibrillations, ECM loss as seen by the lack of GAG staining, and chondrocyte hypertrophy are seen in the earliest stages. As the disease progresses, articular cartilage loss expands from the surface to the tidemark [30].
1.2.2 Subchondral Bone Effects

Alterations to the bone are a direct consequence of the changes in the joint environment during disease progression. In general, these changes include subchondral plate thickening, trabecular bone changes, osteophyte formation, and subchondral bone cysts. According to Wolff’s law, bone adaptation is based on the magnitude and direction of applied force, suggesting that OA changes in the bone are a result of load pattern changes [31]. Early-stage OA is defined in the bone by structural deterioration and increased bone turnover, with several factors implicated. These include microdamage repair, angiogenesis, and increased subchondral plate porosity [32 – 34]. Microdamage may be linear (up to a few hundred microns long) or diffuse (patches of damage), although osteocytes activate resorption in response to linear microcracks only [32]. These microcracks occur in both the bone plate and supporting trabeculae, serving two functions: (1) act as the site of bone remodeling and (2) act as a communication conduit between cartilage and bone. Linear microcracks initiate bone remodeling via osteocyte apoptosis, leading to a high bone turnover. This subsequently leads to a thickened subchondral plate and an advancement of the tidemark due to thickened calcified cartilage, ultimately leading to cartilage thinning [32]. As for angiogenesis, this process occurs as blood vessels from the subchondral bone invade the tidemark in regions of proteoglycan loss, with vascular density increasing as OA progresses. Like the microcracks, this ultimately leads to cartilage thinning as the calcified cartilage advances upwards [33]. Finally, an increase in subchondral plate porosity was demonstrated by Iijima et al. (2016), where rats with surgically induced OA showed an increase in porosity within 2 weeks post-operation. The increase in porosity coincided with adjacent subchondral bone damage and cartilage loss, and was localized to the area of greatest joint loading [34].

Osteophytes are fibrocartilaginous/boney growths localized to joint margins, and are a hallmark of radiographic OA. Animal studies have shown that osteophytes form as a result of periosteal cell proliferation and subsequently differentiation into chondrocytes, which undergo endochondral ossification [21]. Growth factors such as Tumor Growth
Factor β (TGFβ) and Bone Morphogenic Protein-2 (BMP2) are implicated in osteophyte formation, offering a potential therapeutic target [35]. As previously described, Wolff’s law may explain osteophyte growth, as the changing pattern of joint loading may lead to these bony growths in an attempt to maintain joint stability [21].

Further, bone cysts and bone marrow lesions are another commonly seen sign of progressive OA, with bone cysts leading to an increased risk of total knee replacement (TKR) [36]. Bone cysts are composed of fibroconnective tissue filled with fluid, easily seen on MRI, although they may ossify as the disease progresses. Bone cysts have two potential origins: synovial fluid intrusion or bone contusion. The first theory assumes that the breach of the osteochondral junction allows for synovial fluid infiltration, thus leading to bone cysts. In the bone contusion theory, the cysts are due to microcracks and focal bone resorption leading to necrotic lesions [10]. As for bone marrow lesions, they have been observed in healthy patients, although large lesions are associated in knee OA with an increased rate of cartilage loss [37]. This relationship is strengthened by work from Zhao et al. (2010), who utilized MRI technology to demonstrate that bone marrow lesions are preferentially located in areas of cartilage lesions, and the levels of cartilage loss is proportional to bone marrow lesion signal intensity [38]. Overall, the bone is widely affected by OA pathophysiology.

1.2.3 Synovitis

The inflammation of the synovium, termed synovitis, may be caused by joint trauma, infection, and/or joint damage. A failure to resolve this inflammation results in drastic changes to the resident cells, with hyperplasia, angiogenesis, and infiltrates within the synovial membrane. Infiltrating cells, such as T-cells, B-cells, plasma cells, and macrophages, result in destruction of the cartilage matrix and subchondral bone [13]. Synovitis is common in knee OA and its presence is clearly correlated to OA progression. Felson and colleagues (2015) determined that synovitis is an independent risk factor for knee OA development, and Roemer and colleagues (2011) found that effusion synovitis was predictive of cartilage loss [39, 40]. The mechanism of this
increased damage is via synoviocytes and macrophages, which increase the production of pro-inflammatory cytokines and thus catabolic factors in the joint [41]. This has a distinct importance for patients, as knee synovitis carries a 9.2-fold increase in pain severity and is more likely to lead to TKR [42, 43]. The increase in pain could be due to a heightened pain sensitization from increased peripheral nociceptor activity [44]. The synovium is a dynamic structure that requires further focus in OA research.

1.2.4 Supporting Structures Effects

While damage to supporting structures, such as ligaments and meniscus, may cause traumatic knee OA, these structures are also vulnerable to OA damage without trauma. Muscle atrophy is positively correlated with OA disease progression and affects type I and type II muscle fibres [45]. Analysis of the lateral and medial meniscus in OA patients demonstrates fibrocartilage calcification and fraying, cell clustering, and cell hypertrophy [46]. Further, there is an increase in blood vessel density and sensory nerves in the meniscus during OA, serving as a potential source of knee OA pain [47]. As for ligaments, the cruciate ligaments may be affected, with the Anterior Cruciate Ligament (ACL) more so than the Posterior Cruciate Ligament (PCL). Mullaji et al. (2008) compared the ACL and PCL of end-stage knee OA patients, finding that the ACL was most severely affected in patients who had a radiographic OA score of 3 and varus deformity > 15 degrees. These changes are believed to be due to the changes in the force placed on the ACL during flexion/extension, due to altered load mechanics [48]. A later study by Hasegawa et al. (2012) found that changes to the ACL (collagen disorganization, mucoid degeneration, and chondroid metaplasia) was correlated to the presence of OA pathology [49]. The effects of OA on the supporting structures requires further research in order to understand the full spectrum of the disease and how to best therapeutically intervene. Future studies should also look to more rigorously control for different types of OA, particularly post-traumatic OA.
1.3 Risk Factors and Diagnosis

OA diagnosis is based on risk factors, clinical manifestations, and imagining modalities. Risk factors should first be considered, which include age, sex, obesity, occupation, and joint injury. Age is the greatest risk factor for OA, although this could be due to its close relation to the other risk factors, as well as cell senescence [50, 51]. Females are at a higher risk than males to develop knee, hip, and hand OA, with risk increasing at the age of menopause [51]. Obesity is a risk factor for both weight bearing (ex. knee) and non-weight bearing (ex. hand) joints, demonstrating that it plays a more complex role than simply increased joint loading. The adipose tissue most likely increases the secretions of pro-inflammatory factors, leading to a chronic low-grade inflammation [52]. Physically demanding jobs, such as farming or carpentry, increase the risk of lower limb OA due to the associated tasks of squatting, lifting, and prolonged standing [53]. Previous joint injury, such as ligament/meniscal tears, can lead to OA due to an abnormal inflammatory response, discussed more in Section 1.7 [54]. Collecting a comprehensive medical history is an important step in the process of OA diagnosis, but it cannot serve as the sole indicator of the disease.

Clinical manifestations of OA include joint pain that improves with rest, morning stiffness (less than 30 minutes), functional limitations, crepitus, and boney deformity. Joints are typically asymmetrically affected, with the hands, knees, hips, and spine most commonly involved. Physical examinations are used to test the painful range of motion (ROM), ROM limitations, crepitus, and joint instability. There is no specific and sensitive laboratory test for OA, and so an assessment of risk factors and physical examination provide enough information to confidently diagnose OA [55].

Imaging provides an opportunity to visualize any abnormalities within the joint and allows for disease staging. Radiography is the most accessible and thus most commonly used imaging modality for diagnosis [56]. The Kellgren-Lawrence (KL) score is a widely used radiographic classification system ranging from 0 to 4, where 0 represents no OA and 4 is severe OA. The system is based on the presence and
severity of osteophytes, joint space narrowing, sclerosis, and altered bone shape [57].
New methodologies have been used to increase visualization in the knee, where instead of the traditional extended position, a flexed knee with various x-ray beam angles are used [56]. Other, less commonly used forms of imaging include MRI, OCT, and ultrasound. Magnetic Resonance Imaging (MRI) is a useful tool for examining entire joint pathology, as it is accurately able to discriminate articular cartilage, meniscus, and ligaments. Optical Coherence Tomography (OCT) is a real-time analysis of joint tissue, as it is done during arthroscopy and provides cross-sectional images of the cartilage. Finally, ultrasound technology is a cost-effective method of multiplanar imaging that is able to assess dynamic structures in real time [56]. Overall, these imaging modalities are only recommended for clinical use when the OA diagnosis is in doubt due to their high cost, but are an excellent tool for research use [55].

1.4 Pain

Pain is the predominant reason for OA patients to seek medical care, with approximately 39% of OA patients reporting moderate to severe pain, and a 4 times higher rate of avoiding daily activities due to this pain [2]. The early stages of disease are marked by joint pain that improves with rest, but gradually increases over time and may become a constant nocturnal pain. During this time, sleep disruption is often met with mood disorders and fatigue [58]. In fact, it is reported that 60% of OA patients experience trouble sleeping, leading to a generalized fatigue that is strongly associated with worsening mental and physical health [2, 59]. Patients also have an increased risk of developing mood disorders and anxiety, especially within the 35-44 year-old age group, who are 3 times more at risk than those without arthritis [2]. In addition to localized pain, patients report an increase in pain sensitization, which is believed to be due to alterations in nociceptive processing in the peripheral and/or central nervous system. Peripheral sensitization may be due to inflammation affecting local deep somatic nociceptors, while central sensitization is thought to be due to pathological neural signals from the affected joint changing the CNS [58, 60]. Pain sensitization, measured through temporal summation, is significantly associated with symptom
severity but not radiographic severity. Additionally, reversibility of sensitization has been seen following TKR at all sites, and pre-surgical pain sensitivity predicts worse pain outcomes following TKR [60]. Pain sensitization is a complex process that requires further research to establish a definitive pathway and treatment.

Interestingly, radiographic severity and pain severity do not consistently correlate. As seen by Creamer et al. (1999), KL grades demonstrate a poor correlation across three different, validated self-reported measures of pain [61]. A high pain severity with low radiographic severity may be explained by an increase in disease activity, as OA patients with frequent pain have a greater rate of change than pain-free OA patients [62]. So, while pain may not accurately predict radiographic severity, it is more accurately an indicator of disease activity [58, 62]. The exact cause of pain within the joint is most likely not from the cartilage, as it is aneural, leading to the synovium and bone as the tissues of interest. Large bone marrow lesions are associated with greater self-reported pain, with decreased pain as the lesion size decreases [63]. As for synovial inflammation, Baker et al. (2010) reports a 9.2-fold odds increase in knee pain in the presence of synovitis [42].

Molecular and cellular mechanisms of OA pain include signaling through nerve growth factor and cytokines, as well as angiogenesis. Nerve growth factor (NGF) is crucial for nociceceptor development and its blockade shows promising results in animal work [64]. A monoclonal NGF antibody, termed tenazumab, successfully decreases pain, stiffness, and limitations in physical functions in human OA trials, lasting up to 56 weeks [65, 66]. Unfortunately, bone necrosis is found in some patients, leading to a pause in the clinical trials [65, 67]. As for cytokines, some act as pro-nociceptive mediators and may sensitize Aδ- and C-fibres, although human clinical trials targeting some of these have not been successful [56]. Finally, angiogenesis may be targeted through dexamethasone (a corticosteroid), indomethacin (a non-steroidal anti-inflammatory medication), and PPI-2458 (an antiangiogenic fumagillin analog). These compounds successfully reduce pain, synovial inflammation, and synovial angiogenesis in rat models, to varying degrees [47]. Reducing angiogenesis could also play a role in the
subchondral bone, as the increase in subchondral channels containing blood vessels and nerves during OA could be a source of pain and inflammation [58].

1.5 Treatment

There is currently no disease modifying OA drug (DMOAD), so treatment is focused on pain management, with total joint replacement at the end stages. Non-surgical treatment is recommended based on comorbidities, as outlined by OARSI guidelines [68]. Treatment for knee OA specifically is outlined here, as there are some variations depending on disease location. Land based exercise (e.g., yoga, Tai-Chi), weight management, and arthritis education are recommended for all knee OA patients, regardless of comorbidity. Exercise helps relieve pain, and in the case of Tai-Chi is an effective method of increasing neuromuscular communication. Weight management prevents newer or worsening metabolic-OA, and arthritis education assists in patient expectation and disease perception. Other non-pharmacological treatments include aquatic exercise and gait aids (e.g., cane, walking aid), although these are conditional. For example, aquatic exercise specifically has many potential accessibility issues with regards to cost and is not recommended for those with frailty [68].

Pharmacological interventions target pain but not the underlying cause and may include side effects. Paracetamol, although commonly used, does not have a significant efficacy and may lead to liver toxicity, and is therefore not recommended by OARSI [68, 69]. Topical non-steroidal anti-inflammatory drugs (NSAIDs) are strongly recommended if there is no comorbidity, and provide modest benefits with minimal adverse events. Oral NSAIDs are conditionally recommended, with a preference for non-selective NSAIDs with a proton pump inhibitor or selective COX-2 inhibitor. For patients with depression, duloxetine and cognitive behavioural therapy is recommended [68].

Novel treatments for pain management in OA are being examined. Topical capsaicin treatment has been used as an analgesic, so a highly purified and injectable form (CNTX-4975) was established for studies. A multi-centre randomized, double-blind,
placebo-control phase 2 study found that intra-articular injection with CNTX-4975 had clinically relevant pain relief for a prolonged period of time, establishing a rationale for further studies [70]. Studies using biologics have also gained interest in OA treatment, such as those targeting interleukin-1 (IL-1) and tumour necrosis factor (TNF). IL-1 is successfully blocked by lutikizumab, but this compound did not consistently provide pain relief and did not have an effect on synovitis in OA [71, 72]. As for TNF blockage with etanercept, pain relief was observed in an inflammation subgroup after 1 year, but there was no significant relief for the primary group after 2 years. There was no evidence of improved synovitis, but some improvement in bone marrow lesion score. Therefore, it seems that anti-TNF treatments warrant further research, specifically for patients with active inflammation [73].

Intra-articular injections are conditionally recommended for both intra-articular corticosteroids (IACS) and intra-articular hyaluronan (IAHA). IACS potentially provide a shorter term pain relief compared to IAHA, which might provide longer relief and less adverse events, although this is controversial [68]. While commonly used, there is rising evidence that IACS lead to increased radiographic severity, with cartilage loss and joint space narrowing observed [69]. IAHAs, although controversial, are available in multiple forms, varying in production and concentration, and may provide significant pain relief. Overall, IAHAs are well tolerated and adverse events tend to be minimal and mild, with the exception of Synvisc. This version of IAHA therapy has been reported to lead to severe acute inflammatory reactions or pseudo-septic reactions, demonstrating a need to examine each form of IAHA separately [15].

Finally, the most common treatment for end stage OA in the hips and knees is a total joint replacement [74]. Surgery is considered at the end stage when there is a considerable decrease in joint function and increasing pain. In general, current joint prostheses should function for 15 to 20 years, although revision surgery due to instability is possible [55]. Total knee replacement is generally believed to have good rates of patient satisfaction, although there is quite a variance, most likely due to issues in methodology. For instance, only 13% of studies in a systematic review used validated
satisfaction surveys, and 21.2% failed to define a system for satisfaction measurement, making it hard to accurately extrapolate data [75].

1.6 Stratification of OA

OA is a heterogeneous, multifactorial disorder that is generally classified as primary (idiopathic) or secondary (e.g., joint injury), an important distinction for treatment guidelines [76]. Creating distinct phenotypes is difficult as one patient may have multiple risk factors or develop them as the disease progresses. Attempts to define subgroups has varied from etiology, genetics, anatomy, and radiographic phenotypes [77]. A recent systematic review found that studies on knee OA phenotypes focused on a single domain, such as clinical, imaging, or laboratory phenotypes, and are limited as they rarely combined the data. The review did not create distinct subpopulations, but did conclude that pain sensitization, psychological distress, radiographic severity, body mass index, muscle strength, inflammation, and comorbidities are all clinically distinct [78]. Another systematic review of knee OA concludes 6 phenotypes: (1) chronic pain, (2) inflammatory, (3) metabolic syndrome, (4) bone and cartilage metabolism, (5) mechanical overload, and (6) minimal joint disease, but cautioned that it is not clear if these are discrete [79]. The need to stratify in OA research, even with animal models, is stressed by recent work by Maumus et al., (2020), who compared 6 OA models in mice (surgical, aging, inflammatory, obesity, overweight, and metabolic syndrome) on their effect on the TGFβ signaling pathway. The models were lacking a single common gene signature even though the OA scores were similar, demonstrating the importance of clear stratification and avoiding extrapolating information across models [80].

1.7 PTOA

Post-traumatic osteoarthritis (PTOA) is a subtype of OA that develops after joint injury, such as meniscal or ligament injury, typically in the lower extremities [81]. PTOA is estimated to account for 12% of all lower extremity OA cases with costs of about $3
billion USD annually [82]. It may affect any joint, but has the highest incidence in the knee and ankle [81]. This is especially a concern for young athletes, who often sustain a knee injury during high school or college sports. In fact, knee injuries account for 15% of all high school sports-related injuries and 44.6% of surgeries, most commonly due to complete ligament tears [83]. Knee injury increases the risk of OA 4-fold across all ages [84]. Of those, ACL and meniscal injuries carry a high risk of PTOA development, with up to 13% of ACL injuries leading to PTOA, increasing to 21% - 48% for combined ACL and meniscal injuries [85]. Following ACL injury, there is a 1% increase in cartilage lesion risk for each month without surgery [86]. However, another study found that the re-stabilization surgery does not reduce the risk for PTOA, demonstrating that there is more than just biomechanics at play [87].

A proposed mechanism of PTOA is the perpetuation of inflammation, in which some patients are unable to resolve the acute inflammation following joint injury and thus develop chronic inflammation that leads to PTOA [54]. This is supported by research by Sward and colleagues (2012), who collected synovial fluid at various timepoints (up to 23 days) for patients with acute knee injury and assessed the levels of pro-inflammatory cytokines. Levels of IL-1β, IL-6, and TNFα were highest 1 day after injury, then dropping while remaining statistically higher than in healthy knees [88]. The levels of pro-inflammatory cytokines in the synovial fluid may remain elevated up to 6 months post injury, with distinct patterns depending on the stage of inflammation [54, 89]. Certain factors may increase the risk of this chronic inflammation, such as injury severity, repetitive injuries, and an imbalance of pro- and anti-inflammatory factors during the acute and sub-acute period [54]. Work by Heard and colleagues (2013) demonstrates how sustained inflammation increases PTOA using an ovine model that had an ACL transection with immediate reconstructive surgery. They found that cartilage damage and osteophytes occurred 2 weeks post-operatively, but did not progress by 20 weeks post-op. Similarly, pro-inflammatory cytokine levels were initially increased, but normalized by 20 weeks, suggesting the acute inflammatory phase successfully resolved [90]. Exploring both pharmacological (anti-cytokine therapy) and non-
pharmaceutical (exercise) methods to ensure the resolution of inflammation is a worthwhile endeavor in order to reduce PTOA risk [54].

1.8 Inflammation in OA

Inflammation is a physiological response to irritants (e.g., pathogens, physical injury) in the body that leads to swelling, pain, and redness [91]. Inflammation in arthritis is typically associated with rheumatoid arthritis (RA), a systemic disease with joint swelling and damage, although it plays a role in OA as well [92, 93]. OA inflammation is distinctly different from RA in many ways, with OA having lower levels of inflammatory proteins, less pronounced synovitis, and no response to conventional biologics used in RA, to name a few [93]. OA is characterized by a chronic, low grade form of inflammation that involves the innate immune system. Joint trauma, chronic injury, or overuse have the potential to trigger this response through a number of mechanisms, including the pattern recognizing receptor - damage associate molecular pattern (PRR-DAMP) system, the complement system, macrophage response, and cytokines [93].

PRR-DAMPs, the complement system, and macrophages are all part of the innate immune system. PRRs are the first line immune response, recognizing pathogen-associated molecules through cell surface, endosomal, and cytosolic receptors. PRRs are also able to recognize damage-associated molecular patterns (DAMPs), which are endogenous molecules created during tissue damage that signal the immune system the need for repair. PPRs are classified based on location, with the membrane bound toll-like receptors (TLR) the most commonly associated with OA. As for OA-associated DAMPs, there are 4 subgroups; (1) ECM breakdown by-products, (2) plasma proteins, (3) intracellular alarmins, and (4) microscopic crystals [94]. PRR-DAMP activation of TLRs may lead to synovitis and cartilage degeneration, although in vivo testing with agonists yielded mixed results [93].

Similarly, the complement system is the immune system mechanism to recognize pathogens, leading to increased phagocytosis. Activation of the complement system
forms C3 and C5 convertases, which activate the membrane attack complex (MAC) to form transmembrane pores on cells to cause lysis. If levels are sublytic, pro-inflammatory signaling is activated through the mitogen-activated protein kinase, the Janus kinase-signaling transducer and activator of transcription (JAK-STAT) pathway, and the nuclear factor kappa B (NF-κB) pathway [93]. Mouse studies show that a C5 or C6 deficiency attenuates OA and hyper-activation of MAC formation increased OA pathology [95].

Finally, macrophages are implicated as they may be activated by DAMPs and complement, leading to increased cartilage breakdown and subchondral bone changes [93]. The presence of macrophages in the synovium is positively correlated to increased joint pain and stiffness, and a reduced quality of life [16]. This is due to their secretion of pro-inflammatory cytokines and MMPs, which is not balanced by the secretion of anti-inflammatory factors and thus leads to increased cartilage breakdown. Therefore, macrophage ablation has been explored as a therapeutic treatment, with varying success [16]. For example, a mouse model using conditional macrophage depletion, with a high fat diet and surgically-induced OA found a massive infiltration of immune cells into the injured joint and increased concentrations of pro-inflammatory cytokines in the macrophage-depleted mice [96].

1.9 Cytokines

Cytokines are secreted proteins that have a specific effect on the communication between cells that are often redundant, with multiples cytokines having similar functions. They are classically categorized as pro- or anti-inflammatory and are secreted by many cell types, such as synoviocytes, chondrocytes, and macrophages [97]. Pro-inflammatory cytokines are a focus of OA pathophysiology as they increase catabolic factors in the joint, with IL-1 and TNF receiving particular attention [98]. Healthy synovium does have pro-inflammatory cytokines present, although there seems to be a higher presence of anti-inflammatory cytokines that successfully suppress inflammation [17]. An imbalance in this relationship leads to the inflammatory response seen in OA.
In fact, levels of IL-1β and TNF are increased in the synovial fluid, synovium, subchondral bone, and cartilage of the OA joint [99]. IL-1β and TNF will stimulate increased MMP release from chondrocytes and up-regulate ADAMTS-4, leading to collagen breakdown and proteoglycan loss. Additionally, both will cause an increase in other pro-inflammatory cytokines that will further increase MMP and ADAMTS activity, as well as cause the release of nitric oxide (NO) and prostaglandin E2 (PGE2) to enhance MMP activity [98].

In contrast, anti-inflammatory cytokines work to suppress the actions of pro-inflammatory cytokines, like IL-1β and TNF [100]. For example, IL-4 and IL-10 inhibit MMP activity and chondrocyte apoptosis, thus exhibiting a chondroprotective ability. IL-4 specifically is able to decrease the effect of IL-1 and TNF on NO production in vitro, whereas IL-10 stimulates the synthesis of type II collagen and aggrecan [100, 101]. That being said, anti-inflammatory cytokines do not have a primary prophylactic capability but instead are the response to the inflammatory response in OA. Unfortunately, there has not been success in anti-inflammatory based OA treatment [100]. For example, human clinical trials targeting IL-1β and TNF have not been successful even although animal trials have been encouraging. This may be due to the redundant manner of cytokines, an inability of these drugs to penetrate the joint, or testing at the end stage of disease when there may not be a profound effect [98].

1.9.1 IL-15

Interleukin-15 (IL-15) is a pro-inflammatory cytokine that is crucial for natural killer cell (NK) ontogeny and CD8 T cell memory. It is believed that IL-15 is mainly regulated at the post-transcriptional level [102]. There are 3 receptors for IL-15: (1) the specific IL-15Rα, (2) the shared IL-2/IL-15Rβ, and (3) the common IL-15Rγ, binding IL-15, -2, -4, -7, and -19. IL-15 has a high affinity (Ka ≥ 10^{11} M^{-1}) for the IL-15Rα chain, but can only transduce signals in the presence of the IL-15β and γ receptors, for which there is intermediate affinity (Ka = 10^{9} M^{-1}). The IL-15Rβ and γ chains signal via the Janus kinase-1 (Jak1) and Jak3 to activate Signal Transducer and Activator of Transcript 3
(STAT3) and STAT5, respectively. IL-15 has also been shown to activate the NF-κB pathway [100]. IL-15 is widely expressed by monocytes, macrophages, dendritic cells, fibroblasts, epithelial cells, and skeletal muscle [103]. Additionally, IL-15 signaling plays a vital role in the bone turnover process. IL-15Rα ensures efficient osteoblast/osteoclast coupling, as well as determining osteoblast phosphate homeostasis and mineralization capacity [104]. Il15Rα−/− female mice have impaired osteoclast activity and are protected from age related trabecular bone loss when ovariectomized [105]. Indeed, single nucleotide polymorphisms (SNP) of IL-15Rα correlates to total bone volume, as well as cortical bone volume [106]. Overall, IL-15 plays an important role in many body tissues and processes.

The relationship between IL-15 and RA has been more thoroughly investigated, as anti-IL-15 treatment has a potential therapeutic effect, although it may play a role in the chronic, low grade inflammation of OA [107]. IL-15 protein and mRNA levels are reported to be significantly increased in patients with OA compared to the control group [108]. Further, Scanzello et al. (2009) establish that IL-15 protein levels are higher in the synovial fluid during early OA compared to late stage, perhaps demonstrating activation in the early stages of the disease [109]. Interestingly, another study examining the correlation between SNPs in human IL-15Rα and OA found an increased symptom risk by 1.5-fold [110]. Additionally, serum IL-15 levels independently correlate to pain intensity as measured by the Western Ontario McMaster University Osteoarthritis Index (WOMAC), although they do not correlate with KL radiographic severity [111]. The increase of IL-15 in the synovial fluid of OA patients is additionally positively correlated to other pro-inflammatory cytokines, as well as MMP activity [107, 112]. Specifically, Tao and colleagues (2015) demonstrate a strong correlation between MMP-7 serum levels and IL-15 [108]. A recent study determines that the IL-15Rα is present on chondrocytes, and when treated with IL-15 in vitro there is an increase in MMP1 and -3 release, but not in sGAG fragments [110]. Therefore, IL-15 may play a role in OA pathogenesis through an increase in pro-inflammatory activity that additionally creates more MMP activity. This MMP activity may then lead to increased tissue breakdown, and thus more pain and other symptoms.
Successful deletion of the \textit{Il15} gene in Holtzman Sprague-Dawley rats is demonstrated by Renaud and colleagues (2017). Utilizing zinc finger nuclease-mediated genome editing, the second coding exon of \textit{Il15} was targeted with the following sequence CTCAACAGTCACCTTTAACTGAGGCTGGCATCCATG, corresponding to nucleotides 61-97 in rat \textit{Il15} mRNA. The zinc finger nucleases (ZFN) contain a zinc finger protein with specific DNA binding and a \textit{FokI} endonuclease to target DNA breaks. Following site-specific endonuclease activity, the target locus is disrupted and imperfect repair results in functional gene knockouts through frameshift deletion, in this case spanning 7 base pairs. \textit{Il15}^{-/-} rats were created via ZFN microinjection into embryonic day (E)0.5 rats, which were transferred into pseudopregnant rat oviducts. Germline transmission was achieved by backcrossing a founder \textit{Il15}^{+/+} rat with wild-types. \textit{Il15} deficiency was confirmed through western blotting, without any gross morphological effects [113]. This model was established in order to assess the relationship between NK cells and placental development, and so its use in OA research is novel.

1.10 Rationale, Hypothesis, Objective

1.10.1 Rationale

OA is a degenerative disorder with a clear inflammatory component and lack of a successful DMOAD. As such, there is a need to explore novel therapies that suppress the inflammatory mechanisms found in OA. The activity of cytokines is a well-defined area of interest in OA research, with IL-15 showing potential interest as it appears to play a role in increasing local catabolic activity. In order to first establish the role of IL-15 in OA, specifically PTOA, it is beneficial to use a small animal model due to the cost efficiency and potential for genetic engineering. In the present study, OA was surgically induced in \textit{Il15}^{-/-} rats and control \textit{Il15}^{+/+} rats to compare OA progression.

1.10.2 Hypothesis

\textit{Il15}^{-/-} rats will demonstrate a slower progression of PTOA compared to \textit{Il15}^{+/+} rats.
1.10.3 Objectives

Objective 1: Determine if Il15−/− rats have undisturbed baseline joint morphology, in order to make successful comparisons in the PTOA group.

Objective 2: To examine the role of IL-15 in PTOA pathogenesis by comparing disease progression in Il15−/− and Il15+/+ rats.
Chapter 2:
Materials & Methods
2.1 Animals and Genotyping

Dr. Stephen Renaud gifted \( Il15^{-/+} \) Holtzman Sprague-Dawley rats, which were bred inhouse. Rats were housed in a temperature- and humidity-controlled room (20-25 °C, 40-60%), with good ventilation. Water and standard rat chow was freely available. Rats were housed in colony cages and on a standard 12 hour light/dark cycle. All animal experiments were in accordance with the Canadian Council on Animal Care guidelines and were approved by the Animal Use Subcommittee at Western University (2019-029). Rats were genetically modified via ZFN [113]. The process is described in detail in Section 1.9.1, so briefly, the second exon of \( Il15 \) was targeted using zinc finger nuclease-mediated genome editing, causing a 7 base pair frameshift deletion (Fig 2.1.A). A founder rat with a monoallelic \( Il15 \) frameshift deletion was identified and backcrossed to wild type rats in order to achieve germline transmission [113]. A group of \( Il15^{-/+} \) rats were gifted to our group for this experiment and genotyped using extracted DNA from ear biopsies. Four primers were purchased from Sigma-Aldrich to distinguish the wild type and mutant rats by PCR analyses on genomic DNA (Fig 2.1.B-C). \( Il15^{-/+} \) rats were identified by a 152 bp band, \( Il15^{-/-} \) by the 252 bp band, and therefore \( Il15^{-/-} \) by both simultaneously. Male \( Il15^{-/+} \) and \( Il15^{-/-} \) rats were then randomly allocated to either a surgical group (\( N = 15 \)/genotype) or control (\( N = 9 \)/genotype).
Figure 2.1 Genetically modified Holtzmann-Sprague Dawley rats

Holtzmann Sprague-Dawley rats were genetically modified via zinc finger nucleases (ZFN) by Dr. Renaud to delete *Il15* [108]. **A)** The second coding exon of the rat *Il15* gene was targeted via ZFN. Non-coding regions are represented with white boxes, coding regions with black boxes. The red box outlines the ZFN target sequence, which resulted in a 7-nucleotide deletion and subsequent frameshift (red text) for the *Il15*−/− rats. **B - C)** Four primers were used to genotype the rats through PCR. The reverse primer, R1704, was specific to *Il15*+/+ rats and produced a 152-bp band in combination with F1552. The forward primer, F1666, was specific to *Il15*−/− rats and produced a 252 bp-band in combination with R1918.
2.2 Surgery

Garth Blackler performed anterior cruciate ligament transection with destabilizing medial meniscus (ACLT-DMM) surgery on the right knees of 9.5 week old male rats, as described by Appleton et al. (2007) with some modifications [114]. The medial meniscus was destabilized by transecting the medial meniscotibial ligament instead of partially transecting the medial meniscus (Fig 2.2). Surgical anesthesia was induced using 5% Isoflurane, then decreased to 2% for maintenance. Ampicillin (40 mg/kg) was administered subcutaneously as a prophylactic antibiotic, and slow release Buprenorphine (1 mg/mL) was administered subcutaneously as a post-operative analgesic. Age-matched rats that did not have any kind of surgery were used as the controls, termed the Naive group. Weights were measured for the first 4 days post-operatively, and then weekly. All rats were euthanized by asphyxiation with CO₂ at the 8 week timepoint.
Figure 2.2 – Surgically-induced OA

OA was induced through anterior cruciate ligament transection with destabilizing medial meniscus (ACLT-DMM) surgery in male rats. The joint capsule was opened anterior to the medial collateral ligament, followed by transection of the anterior cruciate ligament and the medial meniscotibial ligament. The joint capsule is sutured first, followed by the skin.
2.3 Behavioural Testing

Mechanical allodynia was measured using Electronic von Frey testing (Bioseb, Vitrolles, France). Rats were placed in a Plexiglass box apparatus for 20 minutes of habituation. The plastic von Frey tip was applied to the footpad center of the rat hind paw until there was a pain response, at which point the threshold was electronically recorded. A painful response was counted as anytime the rat withdrew their paw and/or vocalized. Testing was done at baseline (pre-operatively), 4 weeks, and 8 weeks post-operatively.

2.4 Histopathology and Scoring

Right knees were dissected and fixed in 4% paraformaldehyde at 4 °C overnight. An initial decalcification protocol was performed on half of the samples, using Formical-2000™ (StatLab, Baltimore, MD) on coronally bisected joints. Knees were frontally sectioned at 6 µm, following processing and paraffin embedding. This protocol was not effective, and so surface decalcification with Decal Stat™ (StatLab, Baltimore, MD) was performed for each 100 µm of tissue. Due to the variability of surface decalcification and time constraints, a new decalcification protocol was utilized for the remaining samples. Here, Decal Stat™ was used for the first 24 hours, followed by Formical-2000™ as in the previous protocol. Surface decalcification was not necessary for these samples. The centre of the joint was analyzed by staining 3-5 serial sections, 200 µm apart (Fig 2.3.A), stained in 0.04% Toluidine Blue for cartilage damage or with Hematoxylin and Eosin (H&E) for synovitis analysis. Slides were randomized and blinded for scoring by 2 observers.

Toluidine Blue stained sections were analyzed using the Osteoarthritis Research Society International (OARSI) rat histopathologic system [115]. Anatomical landmarks were identified in order to assess section depth and designate quadrants (Fig 2.3.B). Cartilage degeneration, subchondral bone damage, and osteophytes were graded in four knee quadrants - Medial Femoral Condyle (MFC), Medial Tibial Plateau (MTP),
Lateral Femoral Condyle (LFC), and Lateral Tibial Plateau (LTP). As per OARSI guidelines, the cartilage damage is assessed across on a scale from 0 (no damage) to 5 (severe damage). Further, the cartilage in each quadrant was assessed in 3 equal zones, with scores summed in order to represent the total cartilage damage for a maximum total of 15. Zone 1 was labelled as the area adjacent to the meniscus, Zone 2 as the middle area, and Zone 3 being adjacent to the cruciate ligaments (Fig 2.3.C). Similarly, the subchondral bone damage was assessed on a scale from 0 (no damage) to 5 (severe damage), but was not separated into zones. Finally, osteophyte sizes were measured from the osteophyte base to its edge at the thickest point using ImageJ 1.52q by one blinded observer. A corresponding score from 0 – 4 was assigned, as per OARSI guidelines (Fig 2.3.D). For each animal, a score per parameter was assigned based on the mean, median, peak, and summed score for the slides. Within the summed score, there is a maximum score of 75 for total cartilage damage, 25 for subchondral bone damage, and 20 for osteophyte scores.

H&E-stained sections were analyzed using a six parameter synovial scoring system, assessing synovial lining thickness, sub-synovial infiltration, fibrin deposition, vascularization, fibrosis, and perivascular edema [116]. Scoring was done across the Medial and Lateral Parapatellar, Superior, and Inferior compartments with a score of 0 (none) to 3 (severe) for each parameter, resulting in a total of 36 scores per slide (6 parameters across 6 compartments). Final scores were calculated for each animal by calculating the mean score across the 6 compartments per parameter, with the final score representing the sum of the means for a maximum score of 18. For example, to calculate the final synovial lining thickness score for one animal, the mean score from the Medial Parapatellar compartment was calculated from the scored slides. This was then repeated for the remaining compartments. Lastly, the final score was calculated by summing the mean values for each compartment.
Figure 2.3 – OARSI scoring methodology was used to assess OA

Cartilage damage, subchondral bone damage, and osteophytes were assessed using the OARSI scoring system [115]. A) The centre of the joint was collected for scoring, spanning 3 – 5 slides, 200 µm apart. M = medial meniscus, L = lateral meniscus, and A = anterior cruciate ligament. B) Anatomical landmarks were identified in order to assess slide depth and locate quadrants for scoring. C) Cartilage damage was assessed on a scale from 0 – 5 in three equal zones, for a total cartilage damage score out of 15. Zones 1 was adjacent to the meniscus, Zone 2 in the middle, and Zone 3 was adjacent to the cruciate ligaments. D) Osteophyte size was determined by measuring
the osteophyte from the base to its edge at the thickest point, and then a score was assigned from a scale of 0 – 4. Scale bar = 200 µm.
2.5 Statistical Analysis

Statistical analyses were performed in GraphPad Prism v.8.2 and IBM SPSS Statistics v.23. SPSS was used for Cohen’s kappa and Chi-Squared analysis, and Prism for any remaining statistics. Cohen’s kappa was run for inter-rater reliability of cartilage degeneration, subchondral bone damage, and synovitis scoring. The Chi-Squared test of independence with Cramer’s V was run between groups for the presence of osteophytes. Normality was assessed via the D’Agostino & Pearson test. A two-way analysis of variance (ANOVA) with Tukey’s multiple comparison test was run for all OARSI histological scoring. Synovitis scores were analyzed using a one-way ANOVA with Tukey’s multiple comparison test, or the Kruskal-Wallis with Dunn’s multiple comparison test, depending on normality. Weight was compared using a Two-Way ANOVA with repeated measures, and Tukey’s multiple comparison test. Finally, behavioural data were analyzed using a two-way ANOVA, mixed-effects model. P < .05 were considered statistically significant.
Chapter 3:
Results
3.1 General Health and Behavioral Analysis

In order to assess general health following surgery, all animals were weighed daily for the first 4 days post-operation (or timepoint start in the case of the Naive group), then weekly until the end of the 8-week timepoint. There was no significant weight difference between any groups at any timepoints (Fig 3.1). All groups steadily gained weight over time, with a mean increase of 164 g from baseline to week 8. The Il15+/+ PTOA rats were the only group to lose weight during the first 4 days post-op, although it was not significant compared to the remaining groups. The general health of all animals was good, with no adverse events observed following surgery or otherwise.

Mechanical allodynia was measured on a small subset of animals using the Electronic von Frey assay at baseline, 4-, and 8-weeks, represented by paw withdrawal threshold in grams. Values are outlined in Fig 3.2.A. Due to the low N, this data is exploratory only. There was no significant difference in withdrawal thresholds in either of the Naive groups. Within the PTOA groups, both had a significant decrease in withdrawal threshold at 4-weeks post-op, demonstrating an increase in mechanical allodynia. The Il15+/+ PTOA group significantly changed at 8-weeks, returning to baseline levels, while Il15−/− PTOA rats continued to show increased allodynia, but again, this is only exploratory. A more robust N is required in order to make any final conclusions on mechanical allodynia in these groups (Fig 3.2.B).
Figure 3.1 – All groups gained weight similarly over the 8 weeks

Weight, in grams, was measured daily for the first 4 days post-operation, and then weekly for 8 weeks. A Two-Way ANOVA with repeated measures, and Tukey’s multiple comparison was run post-hoc. There was no significant difference between the groups at any timepoint, with all groups steadily gaining weight throughout the experiment. (N = 15 rats/PTOA group, and N = 9 rats/Naive group, p > 0.05, data represented are mean with 95% CI).
Figure 3.2 – Exploratory analyses of effects of PTOA on mechanical alldynia

Electronic von Frey analysis was used to determine changes in mechanical alldynia at baseline, 4-, and 8-weeks. A) Mean paw withdrawal threshold, in grams, is shown per group. B) Exploratory analysis reveals that paw withdrawal threshold was significantly decreased from baseline to 4-weeks in both PTOA groups, but only significantly increased back to baseline at 8-weeks for the II15+/+ PTOA group. Although, a greater N is required in order to make final conclusions. The Naive groups remained unchanged at all timepoints. Data represents results from a Two-Way ANOVA, mixed-effects model with Tukey’s multiple comparison post-hoc. (* p < 0.05, ** p < 0.01, N = 3 rats/PTOA group, and N = 9 rats/Naive group, data represented are mean with SD). C) Plastic von
Frey tip is gradually applied to the middle of the rat hindpaw (red circle) until the rat withdraws their paw and/or vocalizes.
3.2 Control Group

In order to study PTOA in the rat model, OA was surgically induced at 9.5 weeks of age via ACLT-DMM surgery. A sham surgery, where the joint capsule is opened but no further tissue transection takes place, was not performed as we did not want to induce any inflammation in the control group. Instead, we utilized age-matched rats that did not have any kind of surgery to serve as the controls, termed Naive.

The Naive group was first analyzed to determine if there was any OA pathogenesis at that age and to find any differences at baseline (non-surgical) between the genetic strains. Semi-quantitative scoring of histological data demonstrates that there are no significant markers of OA pathogenesis within the Naive group [115, 116]. This includes no significant damage to the articular cartilage, subchondral bone, osteophyte presence/size, or synovitis. Further, the \( \text{Il15}^{+/+} \) and \( \text{Il15}^{-/-} \) Naive groups did not significantly differ (Fig 3.3). The Naive group did have some instances of cartilage or subchondral bone damage in the Lateral Femoral Condyle (LFC) without any obvious traumatic event, for both genetic strains, which are included in the analysis and considered to be variations of normal (Fig 3.4). Overall, the Naive joints demonstrated healthy joint physiology across all 4 quadrants and in both genotypes. With the understanding that the Naive group successfully serves as a control, data is presented comparing the PTOA group.
Figure 3.3 – Representative histological images demonstrate healthy joint tissues in Naive rats
Representative images of toluidine blue-stained paraffin sections of rat knees, demonstrating that the Naive group had no significant differences between genotypes and was overall healthy. Semi-quantitative scoring demonstrates no significant damage to the articular cartilage, subchondral bone, and osteophyte formation. Images were taken at 4X magnification, scale bars represent 200 µm (N = 9 rats/Naive group).
Figure 3.4 – Sample image of tissue damage in Naive rats
Representative image of toluidine blue-stained paraffin section of the variations of normal seen in the Naive group. Instances of cartilage damage with subchondral bone damage was evident in the Lateral Femoral Condyle (LFC) in the absence of a traumatic event in the Naive group. This occurred in both genetic strains and is included in the analysis as a variation of normal. Image taken at 4X magnification, scale bar represents 200 µm (N = 9 rats/Naive group).
3.3 Cartilage Damage after Surgery

Following tissue collection at the end of timepoint, OARSI histological analysis was carried out by 2 scorers, using a semi-quantitative scoring system with a scale from 0 (no damage) to 5 (severe damage) to quantify articular cartilage damage across 4 quadrants. Further, the cartilage in each quadrant was assessed in 3 equal zones, with scores summed in order to represent the total cartilage damage (maximum total of 15) [115]. The weighted kappa score was 0.71, demonstrating substantial rater agreement, according to the Landis & Koch (1977) guidelines [117].

The PTOA groups had significantly higher OARSI scores compared to the Naive groups, which was contained to the medial part of the joint, with the highest scores in the MTP. However, the damage across the articular cartilage was not significantly different between II15+/+ PTOA and II15−/− PTOA rats in the mean, median, peak, and summed scores (Fig 3.5). Zonal analysis revealed that Zone 2 had significantly higher scores, although this was also non-significant when comparing II15+/+ to II15−/− animals. Articular cartilage damage was observed through the loss of GAGs, chondrocyte clustering, and cartilage loss, ranging from surface loss to loss of the deep zone (Fig 3.6). Overall, cartilage damage does not appear to be significantly different in any way between II15+/+ PTOA and II15−/− PTOA rats (Fig 3.7).
Figure 3.5 – Cartilage damage is not significantly different between $\text{II15}^{+/+}$ PTOA and $\text{II15}^{-/-}$ PTOA animals

Animals either underwent ACLT-DMM surgery to induce PTOA or served as control with no surgery (Naive). Semi-quantitative scoring (scale 0 – 5) of toluidine blue-stained paraffin sections was used to determine the extent of cartilage damage in the joint via 4 compartments; medial femoral condyle (MFC), medial tibial plateau (MTP), lateral femoral condyle (LFC), and lateral tibial plateau (LTP). A – D) Two-Way ANOVA with Tukey’s multiple comparison test was run, data represents mean with 95% CI. Mean, median, peak, and summed scores all demonstrate that there was no significant difference between the $\text{II15}^{+/+}$ PTOA and $\text{II15}^{-/-}$ PTOA groups. Across all scores, the PTOA groups scored significantly higher than the Naive groups in the medial compartment. (*** p < 0.001, **** p < 0.0001, N = 15 rats/PTOA group and 9 rats/Naive group).
Figure 3.6 – Representative high magnitude images of articular cartilage damage
Histological images of the cartilage damage found in the PTOA animals. Chondrocyte clustering (red arrow), glycosaminoglycan loss through loss of staining, and cartilage loss is seen. The cartilage loss varies from surface fibrillations (red box) to total loss expanding to the tidemark. Images take at 20X magnification, scale bars represent 100 µm (N = 15 rats/PTOA group).
Figure 3.7 – Representative histological images demonstrate greatest damage in the medial compartment of the PTOA group
Representative images of toluidine blue-stained paraffin sections of rat knees, demonstrating that the PTOA group had the greatest damage in the medial aspect as seen by osteophytes (red arrow), cartilage loss, and subchondral bone damage (red arrow heads). This damage was not significant different between the Il15+/+ PTOA and Il15−/− PTOA rats. Images taken at 4X magnification, scale bars represent 200 μm (N = 15 rats/PTOA group).
3.4 Subchondral Bone and Osteophytes

Subchondral bone damage was assessed similarly to the articular cartilage damage, with a semi-quantitative score from 0 – 5 [115]. There was almost perfect inter-rater agreement in this parameter, with a weighted kappa of 0.86 [117]. Only the medial tibial plateau demonstrated significantly higher scores in the PTOA groups compared to the Naive groups. The mean, median, peak, and summed scores between the IL15+/+ PTOA and IL15−/− PTOA rats was not significantly different in any of quadrants (Fig 3.8.A-D). Subchondral bone damage was observed in the PTOA groups through bone marrow mesenchymal changes, fragmentation of calcified cartilage, marrow chondrogenesis, and articular cartilage collapse (Fig 3.9).

Osteophytes were analyzed by recording the presence or absence by both raters, and subsequently measured by one rater. The presence of osteophytes was analyzed first, revealing a significant relationship between groups and osteophyte presence in the medial compartment. Further analysis found this to be a strong association in both the medial femur and tibia, with the PTOA groups more likely to present with osteophytes (Cramer’s V = 0.64 and 0.60, respectively). In the lateral compartment, the same relationship was present in only the femoral condyle, with a moderate association (Cramer’s V = 0.43; Fig 3.10.A). Examination of the osteophyte size reveals no significant difference between the IL15+/+ PTOA and IL15−/− PTOA rats in the mean, median, peak, and summed scores (Fig 3.10.B-E). Overall, there was no significant difference in the severity of subchondral bone damage between the IL15+/+ PTOA and IL15−/− PTOA rats (Fig 3.7).
Figure 3.8 – Subchondral bone damage is not significantly different between \textit{Il15}$^{+/+}$ PTOA and \textit{Il15}^{-/-} PTOA rats

Animals either underwent ACLT-DMM surgery to induce PTOA or served as a control with no surgery (Naive). Semi-quantitative scoring (scale 0 – 5) was used to determine the extent of subchondral bone damage in the joint via 4 compartments; medial femoral condyle (MFC), medial tibial plateau (MTP), lateral femoral condyle (LFC), and lateral tibial plateau (LTP). A – D) Two-Way ANOVA with Tukey’s multiple comparison test was run, data represents mean with 95% CI. Mean, median, peak, and summed scores all demonstrate that there was no significant difference between the \textit{Il15}$^{+/+}$ PTOA and \textit{Il15}^{-/-} PTOA groups. Across all scores, the PTOA groups scored significantly higher than the Naive groups only in the MTP. (**** $p < 0.0001$, N = 15 rats/PTOA group and 9 rats/Naive).
Figure 3.9 – Representative image of subchondral bone damage in PTOA rats

Histological images of the articular cartilage collapse/infiltrating into the subchondral bone. The collapse varies from a mild break in the tidemark, with some non-calcified cartilage infiltrating into the bone, to the most severe, where the cartilage has collapsed to a depth greater than 250 µm with increased mesenchymal changes to the bone marrow. Images take at 20X magnification, scale bars represent 100 µm (N = 15 rats/PTOA group).
Figure 3.10 – Osteophyte size is not significantly different between Il15+/+ and Il15−/− animals

Animals either underwent ACLT-DMM surgery to induce PTOA or served as control with no surgery (Naive). Osteophytes were analyzed for absence/presence and size in the 4 compartments; medial femoral condyle (MFC), medial tibial plateau (MTP), lateral femoral condyle (LFC), and lateral tibial plateau (LTP). A) Presence of osteophytes was determined and agreed upon by two scorers. Chi-squared analysis with Cramer’s V established that the PTOA groups were strongly associated with osteophyte presence in the medial compartment, and moderately associated in the LFC, with no significance in the LTP. (MFC = X² (3, N = 45) = 18.24, p < 0.0001, Cramer’s V = 0.64, MTP = X² (3, N = 45) = 16.38, p < 0.005, Cramer’s V = 0.60, LFC = X² (6, N = 45) = 16.90, p < 0.05, Cramer’s V = 0.43, LTP = X² (3, N = 45) = 7.05, p > 0.05). B – E) Two-Way ANOVA
with Tukey’s multiple comparison test was run, and data represents mean with 95% CI. Mean, median, peak, and summed scores all demonstrate that there was no significant difference between the \textit{Il15}^+/+ PTOA and \textit{Il15}^-/- PTOA groups. Mean, median, and peak represents osteophyte size, while the summed score represents the designated OARSI score ($p > 0.05$, $N = 15$ rats/PTOA group and 9 rats/Naive group).
3.5 Synovitis

Synovitis was similarly assessed using a semi-quantitative 6 parameter scoring system [116]. A score from 0 (none) to 3 (severe) was given across 6 compartments (medial and lateral parapatellar, superior, and inferior compartments) for 6 parameters; (1) synovial lining thickness, (2) sub-synovial infiltration, (3) surface fibrin deposition, (4) vascularization, (5) fibrosis, and (6) perivascular edema. Preliminary inter-rater analysis reveals substantial to almost perfect agreement, with a weighted kappa ranging from 0.67 to 0.85 across the six parameters (Table 3.1) [117]. There was no significant difference between the $II15^+/+$ PTOA and $II15^-/-$ PTOA rats across all six parameters (Fig 3.11). When comparing the PTOA to Naive group, only the sub-synovial infiltrate and surface fibrin scores are significant, but again there is no difference between genetic strains (Fig 3.12). Overall, synovitis is not significantly different between the $II15^+/+$ PTOA and $II15^-/-$ PTOA rats.

### Table 3.1 Inter-rater Agreement

<table>
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<th>Parameter</th>
<th>Weighted Kappa</th>
<th>Agreement</th>
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<td>Synovial lining thickness</td>
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<td>Substantial</td>
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<tr>
<td>Sub-synovial infiltration</td>
<td>0.77</td>
<td>Substantial</td>
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<tr>
<td>Fibrin deposition</td>
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<td>Substantial</td>
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<td>Vascularization</td>
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<td>Fibrosis</td>
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<td>Almost Perfect</td>
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<td>Perivascular edema</td>
<td>0.85</td>
<td>Almost Perfect</td>
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Rats either underwent ACLT-DMM surgery to induce PTOA or served as control with no surgery (Naive). Semi-quantitative scoring was used to assess synovitis across 6 parameters, ranging from 0 (none) to 3 (severe), measured in 6 zones ( medial and lateral parapatellar, superior, and inferior compartments). A One-Way ANOVA with Tukey’s multiple comparison test or the Kruskal-Wallis with Dunn’s multiple comparison test was run, depending on normality. Data represents mean with 95% CI. Analysis
reveals that the PTOA group had scored significantly higher for sub-synovial infiltration and surface fibrin, although there was no significant difference between genetic strains. The remaining 4 parameters were non-significant across all groups (* p < 0.05, N = 15 rats/PTOA group and 9 rats/Naive group).
Figure 3.12 – Representative images of synovitis in the PTOA group

Histological images of the synovitis, as demonstrated by surface fibrin and sub-synovial infiltration, found in the PTOA animals. Healthy synovium (first box) is presented as a comparison and represents Naive tissue. Surface fibrin (red arrow) and sub-synovial infiltration (red arrowheads) was found significantly more in the PTOA group compared to the Naive, but was not different between genetic variants. Images take at 20X magnification, scale bars represent 100 µm (N = 15 rats/PTOA group and N = 9 rats/Naive group).
Chapter 4:
Discussion
Cytokine activity is a well-defined research area in OA, offering many potential therapeutic targets to be explored. Interleukin-15 (IL-15), a pro-inflammatory cytokine, has been shown to have increased levels in OA and can potentially increase catabolic activity within the joint. Our study utilized a Holtzmann Sprague-Dawley male global Il15−/− model to study the roles of IL-15 in PTOA progression. Our work demonstrated that Il15−/− rats present with normal joint morphology and display similar PTOA progression as their Il15+/+ counterparts. Through the analysis of cartilage damage, subchondral bone damage, osteophyte formation, exploratory behavioural data, and synovitis we did not see any significant differences between genotypes, suggesting that IL-15 does not play a vital role in PTOA progression in this rat model. These data do not support our initial hypothesis.

The use of Il15−/− rats in OA research is novel, and so we first had to establish that mutant rats have normal joint morphology in the absence of surgery. The whole-body (global) Il15 knockout was generated by Renaud and colleagues (2017) and confirmed through western blotting [113]. They did not observe gross morphological defects, but the model was created to examine placental development, and so joint health was not studied. The Naive group, which did not undergo PTOA-inducing surgery, was examined first. Our results did not find any evidence of disturbed joint morphology in the Il15−/− rats across all parameters, compared to the Il15+/+. This includes no statistically significant evidence for pain sensitization, cartilage damage, subchondral bone damage, osteophyte formation and size, and synovitis. Overall, the Il15−/− rats had healthy knee joints that were comparable to the Il15+/+ rats in all ways. Interestingly, there was some instances of disturbed subchondral bone without obvious trauma in the Naive group, but this occurred in both genetic strains. This damage was generally observed in the LFC, which was minimally damaged in the PTOA groups. As this was observed equally in both Il15−/− and Il15+/+ rats without a clear triggering event, the data was included in the analysis as a variation of normal. Indeed, these variations did not result in any significant differences when analyzing the subchondral bone.
Weight was recorded as a measure of general health, especially for the PTOA group following surgery. There was no significant difference in weight at any timepoint between all groups, similar to the findings from Renaud et al. (2017) with their female rats [113]. Even though the $Il15^{+/+}$ PTOA rats lost weight during the first 4 days post-op, this loss was not significant, and they continued to gain weight in the weeks following. Interestingly, the $Il15^{-/-}$ rats, in both the PTOA and Naive groups, consistently weighed less than their $Il15^{+/+}$ counterparts, although not enough to reach statistical significance. $Il15^{-/-}$ mice have varied in their reported weight differences in the literature, for example Kennedy et al. (2000) reported that $Il15^{-/-}$ mice were not significantly different in body weight compared to $Il15^{+/+}$ mice, but another study reported their male $Il15^{-/-}$ mice weighed less [118, 119]. Similarly, the mouse model of $Il15R\alpha^{-/-}$ (IL-15 receptor alpha deficient mice) has conflicting reports of weight, either reporting similar weights or a decrease in weight in mutants compared to controls [120, 121]. Interestingly, $Il15^{-/-}$ mice on a high fat diet are resistant to diet-induced weight gain due to increased thermogenic capacity in brown and beige fat cells [122]. Our male $Il15^{-/-}$ rats appear to trend towards weighing less, perhaps due to a similar increased thermogenic capacity, although not enough to reach statistical significance. Another explanation could be the importance of IL-15 signaling in bone turnover, as IL-15 signaling is critical for osteoblast/osteoclast coupling and the mineralization capacity of osteoblasts, as seen in $Il15R\alpha^{-/-}$ mice [104]. Perhaps the decrease in bone mineralization contributed to the weight loss trend in our $Il15^{-/-}$ rats.

In order to assess PTOA progression, surgery to transect the anterior cruciate ligament and destabilize the medial meniscus (ACLT-DMM) was utilized in both $Il15^{-/-}$ and $Il15^{+/+}$ rats. Collection at the 8-week timepoint after surgery revealed that the surgery successfully induced PTOA, as this group had statistically higher scores for cartilage damage, subchondral bone damage, and osteophyte presence compared to the Naive group. As expected, the damage was generally contained to the more weight bearing medial compartment. There is also evidence of synovitis developing in the PTOA group, as they scored significantly higher for sub-synovial infiltration and surface fibrin deposition, compared to the Naive group. Further, behavioural data found an increase
in pain sensitivity in the PTOA groups, although this is exploratory (N = 3 rats/PTOA group, N = 9 rats/Naive group). Our exploratory data followed the expected pattern, where pain sensitization was greatest around 4 weeks, and then slightly declined and plateaued [123]. The Il15+/− rats had a significant reduction in pain sensitization from 4- to 8-weeks, but the Il15−/− rats did not, but further data including a much larger N is required in order to make any conclusions.

Synovitis, as measured through 6 parameters, was observed in the PTOA group through sub-synovial infiltration and surface fibrin deposition. Previous studies from the Appleton lab demonstrated synovial hyperplasia at 4-weeks and surface fibrin at both 4- and 12-week timepoints, although there was no data for 8-weeks (unpublished). Our data follows the expected pattern, where surface fibrin continues to be present at all timepoints. Additionally, the sub-synovial infiltration we found follows the expected trend, as this parameter was increased at 4-weeks and then decreased by 12-weeks, although not significantly. Taken together, these data suggest sub-synovial infiltration begins increasing as early as 4-weeks, reaches significant levels by 8-weeks, and then decreases at 12-weeks. Overall, ACLT-DMM surgery successfully induced mid-stage PTOA.

Inconsistent with our hypothesis, there was no significant difference between the Il15−/− and Il15+/− PTOA rats, indicating that IL-15 may not play a vital role in rat PTOA pathophysiology. This finding is also inconsistent with previous studies that found a relationship between IL-15 activity and OA, which may be due to a few factors. Firstly, it is possible that the rodent model is not ideal for translating studies in IL-15, as current literature on IL-15 and OA has exclusively been reported from human studies [108-112]. This is one of the limitations with small animal research, although the cost-effective and ease of use provides an excellent opportunity to explore therapeutic targets. As such, while there could be translational issues between the studies, other limiting factors should be considered first. For example, our study examined only the 8-week timepoint, but the activity of IL-15 may be more robust at an earlier or later stage of disease. Work by Scanzello et al. (2009), comparing early and late stage IL-15 levels, would suggest
that IL-15 is more active during the early stage of disease [109]. Comparatively, studies measuring IL-15 levels in OA patients tend to utilize patients at the end stage of the disease, due to the convenience of tissue collection during arthroplastic surgery. Elevated levels of IL-15 in these patients could suggest that it continues to play a role even at the end-stage of disease [108, 112]. Our model could benefit from the addition of early and late stage PTOA in order to further explore this relationship.

Another pitfall could be due to differing OA phenotypes. The current literature examining IL-15 and OA has excluded patients with a history of traumatic joint injury, thus excluding PTOA, which could be another reason for the lack of significance between groups [108-112]. IL-15 may play a more vital role in other OA phenotypes, such as primary OA, as seen in human studies, or potentially in metabolic OA. Research utilizing \( \text{Il}15^{-/-} \) mice on a high fat diet revealed an important role for IL-15 in metabolic syndrome, as it seems to promote chronic inflammation in adipose tissue [122]. Perhaps the inflammatory role of IL-15 is more substantial in metabolic OA than in PTOA. Finally, the redundant and pleiotropic nature of cytokines could explain why the absence of IL-15 did not cause a change in PTOA progression. This is partly due to the ability to signal through multiple receptor complexes and shared receptors; for example, IL-15 has a shared \( \beta \) receptor with IL-2 and a common \( \gamma \) receptor, shared by 5 interleukins [102, 124]. This redundant nature may be a reason that therapy singularly targeting IL-1\( \beta \) or TNF\( \alpha \) has not been effective, and significant slowing of OA progression could require a combination therapy [125]. Additionally, the activity of IL-15 on catabolic factors, like MMPs, may be more indirect, as suggested by recent work by Warner and colleagues (2020). Chondrocytes treated with IL-15 \textit{in vitro} demonstrate a delayed release of MMP-1 and -3, compared to TNF\( \alpha \), which may point to an indirect effect of IL-15 on these MMPs [110]. Therefore, the action of IL-15 to increase catabolic activity in the joint may be taken over by another cytokine when IL-15 is absent, especially if this activity is indirect.
Limitations and Future Directions

It is important to consider the limitations involved in this thesis. Many of these limitations were due to the laboratory shutdown during the COVID-19 pandemic, which intervened with completion of additional in vivo experiments. Within our study, the behavioural data is exploratory due to the small N in the PTOA group. While our work did follow the expected pattern of pain sensitivity following surgery, it is not clear whether there are significant differences between the groups and genotypes. Further, we only analyzed one pain measure, which while helpful, does not encompass the multifaceted nature of OA pain. Future work should include further pain measures, such as mechanical analgesia or thermal hyperalgesia. Our work also lacked immunohistochemistry (IHC) or other molecular data, although there was an attempt to demonstrate IL-15 presence in the joint through IHC. Since IL-15 is mandatory for NK cell maturation, we targeted perforin, a pore-forming protein found in NK cells, as a surrogate marker for IL-15 activity. Unfortunately, this is a non-specific antibody whose target is also found in T-lymphocytes, so we were unable to obtain convincing data to effectively compare IL-15 activity. Future work would benefit from an optimized method of IL-15 detection in the joint in order to assess the expression of IL-15 in the rodent joint. As previously discussed, focusing on one timepoint and one OA phenotype only could also be a limitation, and so studies involving multiple timepoints and perhaps a metabolic or aging OA model could be beneficial to exploring the role of IL-15 in OA.

Other limitations include the use of a global (whole-body) knockout rodent model, which limits our confidence in the effects of IL-15 on the joint. As it is widely expressed, there could have been off target effects or compensatory events that interfered with our study. This could be solved by utilizing tissue-specific Cre drivers to target only the cartilage or subchondral bone, although these approaches are much less established in rats than in mice. Additionally, our work only examined male rats, although sex-related differences are possible. In fact, OA incidence, progression, and severity are higher in post-menopausal females, demonstrating that sex hormones could be chondroprotective pre-menopause [126]. In addition, male mice develop more severe surgically-induced OA.
compared to females [127]. It would therefore be beneficial to examine \( \text{Il}15^{-/-} \) female rats as well to explore any sex-related differences.

Further, the use of a solely \textit{in vivo} model limits the scope of our work in understanding the mechanisms of IL-15 in a more complex manner. An \textit{in vitro} model studying primary rat or human synoviocytes that are treated with IL-15 would be an interesting study, demonstrating the effects and mechanism of IL-15 in the synovium. Finally, our ACLT-DMM model is invasive and does not perfectly mimic the real world injuries associated with PTOA. In our model, the joint capsule must first be transected in order to access the ACL and meniscus, which would normally remain undisturbed during joint trauma. In order to successfully transect both structures some blood has to be cleared, although every attempt is made to clear as little as possible and suture the joint capsule closed with the blood present. This is done in order to minimize any unintended effects on the progression of PTOA, although there may still be some effect. Future work could utilize a non-invasive model, where the ACL is ruptured via tibial compression in order to induce PTOA [128, 129].

Overall, while our work did not demonstrate a significant difference in IL-15 and PTOA, there are still many avenues to explore with IL-15 to elucidate its role in OA pathogenesis.
Chapter 6:
References


interleukin-15 differentiates early from end-stage disease. *Osteoarthritis Cartilage* 17, 1040-1048.


Appendix A:

Animal Protocol Notice of Approval
From: eSirius3GWebServer
Subject: eSirius3G Notification -- 2019-029 Annual Renewal Approved
Date: June 1, 2020 at 11:22 AM

2019-029:3:
AUP Number: 2019-029
AUP Title: TGFalpha/EGFR signaling in osteoarthritis
Yearly Renewal Date: 06/01/2021

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2019-029 has been approved by the Animal Care Committee (ACC), and will be approved through to the above review date.

Please at this time review your AUP with your research team to ensure full understanding by everyone listed within this AUP.

As per your declaration within this approved AUP, you are obligated to ensure that:

1) Animals used in this research project will be cared for in alignment with:
   a) Western’s Senate MAPPs 7.12, 7.10, and 7.15
      http://www.uwo.ca/univsec/policies_procedures/research.html
   b) University Council on Animal Care Policies and related Animal Care Committee procedures
      http://uwo.ca/research/services/animalethics/animal_care_and_use_policies.html

2) As per UCAC’s Animal Use Protocols Policy,
   a) this AUP accurately represents intended animal use;
   b) external approvals associated with this AUP, including permits and scientific/departmental peer approvals, are complete and accurate;
   c) any divergence from this AUP will not be undertaken until the related Protocol Modification is approved by the ACC;
   d) AUP form submissions - Annual Protocol Renewals and Full AUP Renewals - will be submitted
      and attended to within timeframes outlined by the ACC:  http://uwo.ca/research/services/animalethics/animal_use_protocols.html

3) As per MAPP 7.10 all individuals listed within this AUP as having any hands-on animal contact will:
   a) be made familiar with and have direct access to this AUP;
   b) complete all required CCAC mandatory training (training@uwo.ca); and
   c) be overseen by me to ensure appropriate care and use of animals.

4) As per MAPP 7.15,
   a) Practice will align with approved AUP elements;
   b) Unrestricted access to all animal areas will be given to ACVS Veterinarians and ACC Leaders;
   c) UCAC policies and related ACC procedures will be followed, including but not limited to:
      i) Research Animal Procurement
      ii) Animal Care and Use Records
      iii) Sick Animal Response
      iv) Continuing Care Visits

5) As per institutional OH&S policies, all individuals listed within this AUP who will be using or potentially exposed to hazardous materials will have completed in advance the appropriate institutional OH&S training, facility-level training, and reviewed related (M)SDS Sheets,  http://www.uwo.ca/hr/learning/required/index.html

Submitted by:
on behalf of the Animal Care Committee
University Council on Animal Care

The University of Western Ontario
Animal Care Committee / University Council on Animal Care

http://www.uwo.ca/research/services/animalethics/index.html

*** THIS IS AN EMAIL NOTIFICATION ONLY. PLEASE DO NOT REPLY ***
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