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# The effects of specialized pro-resolving mediators and sex on post-traumatic osteoarthritis development

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BOSTON UNIVERSITY

ARAM V. CHOBANIAN & EDWARD AVEDISIAN SCHOOL OF MEDICINE

Thesis

**THE EFFECTS OF SPECIALIZED PRO-RESOLVING MEDIATORS AND SEX  
ON POST-TRAUMATIC OSTEOARTHRITIS DEVELOPMENT**

by

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B.S., Loyola University Chicago, 2021

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**THE EFFECTS OF SPECIALIZED PRO-RESOLVING MEDIATORS AND SEX  
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**JULIE MEKHAIL**

**ABSTRACT**

**INTRODUCTION:** Anterior cruciate ligament (ACL) tears lead to joint inflammation and osteochondral degeneration culminating in posttraumatic osteoarthritis (PTOA). Inflammation resolution is actively modulated by specialized pro-resolving mediators (SPMs), fatty-acid derivatives with anti-inflammatory, analgesic, and regenerative properties. It is unknown whether SPMs play a role in PTOA. We investigated whether pro-resolving pathways are induced in a mouse model of ACL injury, focusing on maresin1 (MaR1) and resolvin D1 (RvD1), two omega-3-derived SPMs. We also investigated the sexual dimorphism in PTOA severity following ACL injury.

**METHODS:** Eight-week-old C57BL6/J mice underwent ACLT. Joint tissues were harvested from surgical and control unoperated contralateral knee at multiple time points. Periarticular synovial tissue expression of pro-inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ ), SPM biosynthetic enzyme (12/15Alox) and receptors (LGR6, FPR2), and synovial fluid levels of MaR1 and RvD1 were measured by qPCR and ELISA, respectively. OARSI scoring was performed by blinded observers.

**RESULTS:** OARSI scoring and  $\mu$ CT scoring at 8 weeks post-injury demonstrated that ACLT induces PTOA. Thus, we assessed the acute inflammatory and pro-resolving responses to ACL injury. ACLT induced acute inflammation with increased cellular

infiltrate and induction of TNF- $\alpha$ /IL-1 $\beta$  expression in the first week post-injury. Resolvin pathways were also induced. Joint injury induced local production of synovial fluid MaR1 and RvD1, with levels peaking at 1 week after ACLT. Moreover, 12/15Alox and LGR6/FPR2 expression increase post-injury. Despite induction of SPMs, evidence of chronic inflammation persisted through week 8.  $\mu$ CT and histology results also showed that male mice showed more severe PTOA than female mice.

**CONCLUSION:** Joint injury in ACLT acutely results in both inflammation and induction of the SPMs Mar1 and RvD1, as well as their biosynthetic enzymes and receptors. However, induction of these pro-resolving pathways does not completely resolve inflammation, which remains evident for months post-injury. Although the endogenous resolution response after injury is insufficient to restore tissue homeostasis and prevent damage, the existence of these pathways in the joint suggests that exogenous SPMs could be a potential intervention to reduce inflammation and damage after joint injury. Furthermore, results also suggest the presence of some protective mechanism against PTOA development in female mice in comparison to male mice.

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## LIST OF ABBREVIATIONS

ACL	Anterior cruciate ligament
ACLT	Anterior cruciate ligament transection
CCL2	Chemokine (C-C motif) ligand 2
Ct	Cycle threshold
CXCL1	Chemokine (C-X-C motif) ligand 1
$\mu$ CT	Micro-computed tomography
ELISA	Enzyme-linked Immunosorbent Assay
Fpr2	Formyl-peptide receptor-2
GPCR	G-protein coupled receptor
IACUC	Institutional Animal Care and Use Committee
ICC	Interclass correlation coefficient
IP	Intraperitoneal
KLF4	Krüppel-like factor 4
KO	Knockout
LGR6	Leucine-rich repeat-containing G-protein coupled receptor 6
Mar1	Maresin 1
NSAIDs	Nonsteroidal anti-inflammatory drugs
OA	Osteoarthritis
OARSI	Osteoarthritis Research Society International
PBS	Phosphate-buffered saline

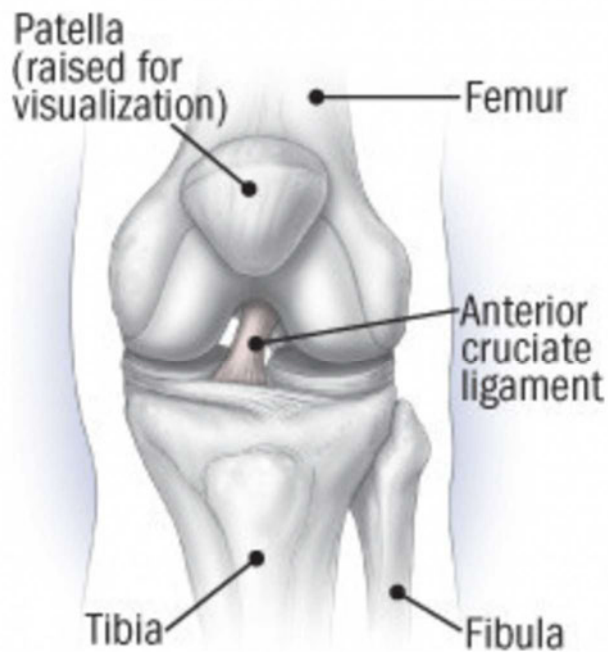
PCL	Posterior cruciate ligament
PFA	Paraformaldehyde
PTOA	Post-traumatic osteoarthritis
qPCR	Quantitative polymerase chain reaction
RvD1	Resolvin D1
SPM	Specialized pro-resolving mediator
TNF	Tumor necrosis factor
WT	Wild type
5Alox	Arachidonate 5-lipoxygenase
12/15Alox	Arachidonate 12/15-lipoxygenase

## **INTRODUCTION**

Anterior cruciate ligament (ACL) tears are among the most common orthopedic injuries, affecting hundreds of thousands of people every year. These injuries can have significant long-term consequences, including the development of post-traumatic osteoarthritis (PTOA), with ACL tears as the most common cause of PTOA in the United States. PTOA occurs when chronic inflammation or instability in the joint leads to the degradation of cartilage and other joint tissues. It is believed and shown in previous studies that ACL tears cause chronic inflammation, which we hypothesize leads to the ongoing damage and inflammation that results in PTOA (Lattermann, C. et al., 2018). Recent research has suggested that interventions aimed at promoting inflammation resolution rather than simply reducing inflammation may be more effective at preventing PTOA, and this thesis will explore that possibility in more detail (Serhan and Levy, 2018).

### **Anterior Cruciate Ligament**

The ACL is a vital structure in the knee joint. The ACL is formed of dense connective tissue that provides stability by connecting the femur and tibia (Figure 1) (Shah et al., 2010).



**Figure 1: The anatomy of the anterior cruciate ligament.** Anatomical representation of the knee joint with visualization of the anterior cruciate ligament (Harvard Health, 2020).

The ACL, together with the posterior cruciate ligament (PCL), forms the main source of knee stabilization. The ACL plays a crucial role in resisting anterior tibial translation, preventing the tibia from sliding forward relative to the femur (Shah et al., 2010). Additionally, it helps to restrain internal tibial rotation and excessive knee extension, ensuring the stability of the knee joint during movements such as jumping, pivoting, or decelerating. The ACL also has a protective function for the menisci of the knee, which can be damaged by the strong forces generated during high-intensity physical activities (Woo et al., 1991). Unfortunately, ACL injuries are prevalent among athletes, particularly those who engage in sports that require frequent jumping, cutting,

and pivoting movements (Hootman, J. M., and Dick, R. 2007). The incidence of ACL injuries has increased significantly over the past two decades, mainly due to the rising popularity of sports and military training, leading to a higher frequency of ACL injuries in younger populations (LaBella CR et al., 2014). As a result of the increased incidence of ACL injuries, continued research and innovation in the field of ACL injury management is essential to meet the growing demand for effective and sustainable treatments.

Injury to the anterior cruciate ligament can have serious consequences that go beyond the initial damage to the ligament itself. In fact, the injury often triggers a cascade of events that involve bleeding into the affected joint, tissue damage, and inflammation of not only the joint but also the surrounding tissues (Lattermann, C. et al., 2018). This inflammation can lead to prolonged pain, swelling, and stiffness in the affected joint. The current primary treatment for ACL injuries involves stabilization of the joint through surgical reconstruction of the ACL (Raines et al., 2017). Other patients opt for a more conservative treatment involving physical therapy. Treatment options for pain related to ACL injury include short-course anti-inflammatory drugs, corticosteroids, nonsteroidal anti-inflammatory drugs (NSAIDs), and viscosupplementation (Raines et al., 2017).

### **Post Traumatic Osteoarthritis**

Post-traumatic osteoarthritis (PTOA) is a chronic, progressive joint disease that often develops following injury to the bone or joint (Gong et al., 2023). The damage caused by the insult leads to a cascade of cellular and molecular events, including inflammation, cartilage and bone degradation, and aberrant joint remodeling (Lattermann,

C. et al., 2018). These processes can eventually lead to the development of osteoarthritis in the affected joint. PTOA is a major public health concern, as it is most commonly seen in younger adults (Gong et al., 2023). In addition to the debilitating pain and stiffness that PTOA patients experience, the disease can also have a significant economic impact due to the need for costly medical interventions and reduced work productivity.

ACL injuries are the most common cause of knee PTOA with over 50% of ACL-injured patients developing PTOA within 10 to 20 years after injury (LaBella CR et al., 2014). According to clinical data, individuals that suffer an ACL injury are ten times more likely to acquire degenerative knee arthritis than uninjured individuals (LaBella CR et al., 2014). Surgical reconstruction or replacement of the ACL can help restore the stability of the knee, however; this intervention is not sufficient to prevent the development of PTOA (Bigoni et al., 2013). The low levels of chronic inflammation that follow an ACL injury are not mitigated by surgical reconstruction, therefore, this inflammation is thought to cause substantial degenerative modifications to the affected bone and cartilage (Lattermann, C. et al., 2018). Certain inflammatory cytokines, such as tumor necrosis factor (TNF), IL-6, IL-8, and IL-17, are increased following an ACL injury (Lattermann, C. et al., 2018). These inflammatory mediators are known to cause cartilage degradation, possibly contributing to the PTOA pathogenesis.

Furthermore, structural changes play a critical role in the pathogenesis of PTOA. Following an injury, there is damage to the joint structures such as cartilage, subchondral bone, synovial membrane, ligaments, and menisci. This damage triggers a cascade of events including chondrocyte apoptosis and abnormal remodeling of the extracellular

matrix. As a result, the joint loses its normal structural integrity, leading to the development of PTOA. Over time, these structural changes worsen, resulting in pain, stiffness, and loss of function. Studies have shown that levels of CTXII, a known biomarker of knee osteoarthritis, are also shown to be elevated in patients who recently experienced an ACL injury (Lattermann, C. et al., 2017). CTXII are fragments of type II collagen that are released during the breakdown of articular cartilage, a symptom of PTOA.

### **Resolution of Inflammation**

With most injuries, there is an inflammatory response, followed by a resolution back to baseline levels. Following ACL tears, however, there seems to be a lack of complete resolution, leading to chronic, low-level inflammation (Bigoni et al., 2013). This chronic inflammatory response is thought to drive the development of PTOA through the damage of cartilage and bone and the persistence of pro-inflammatory cells and cytokines.

Resolution of inflammation, which was thought to be a passive process but is now known to be an active process, is partly modulated by specialized pro-resolving mediators (SPMs) (Dalli and Serhan, 2019). SPMs are omega-3 and omega-6-polyunsaturated fatty acid-derived lipid molecules with several pro-resolutive effects such as anti-inflammation, pain relief, and tissue repair (Serhan et al., 2015). There are several classes of SPMs (Resolvins, Maresins, Protectins, and Lipoxins) based on their chemical structure, their source (i.e., which fatty acid they are derived from), and their specific biological effects. In particular, Resolvin D1 (RvD1) is an SPM that inhibits

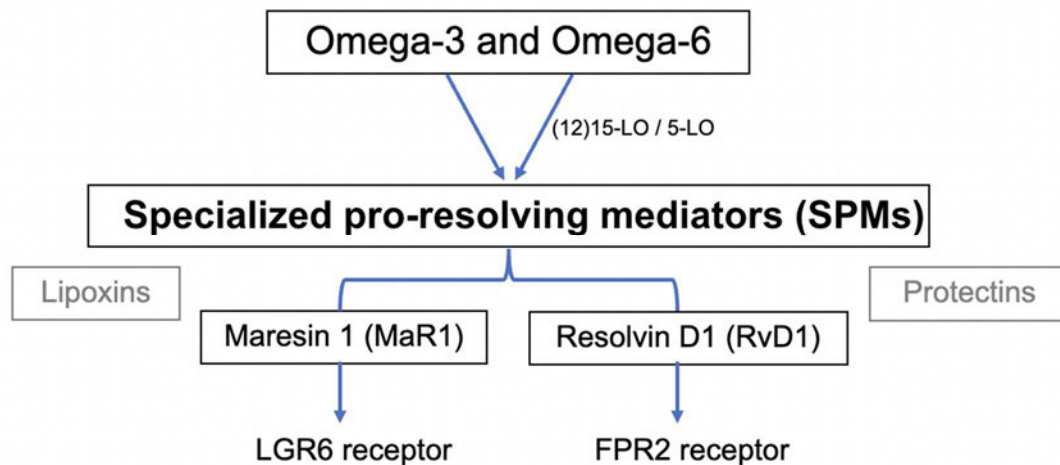
granulocyte trafficking, dampens pro-inflammatory signaling, promotes efferocytosis, and contributes to the switch from M1 phenotype to M2 phenotype macrophages (Figure 2) (Fullerton, J., Gilroy, D., 2016). Similarly, Maresins are SPMs that inhibit granulocyte trafficking and promote efferocytosis (Figure 2) (Fullerton, J., Gilroy, D., 2016). Mar1 binds to a G-coupled protein coupled receptor (GPCR) named LGR6 and RvD1 binds to a GPCR called FPR2 (Figure 2).

Oftentimes, there is confusion about the differences between anti-inflammation and pro-resolution. Resolution pathways not only assist in the termination of inflammatory responses but also direct the shift from innate to adaptive immunity (Dalli and Serhan, 2019). Diseases that are associated with chronic inflammation might stunt the resolution of inflammation by pathologically silencing pro-resolution pathways (Fullerton, J., Gilroy, D., 2016). This concept suggests that there might be an opportunity in mitigating chronic inflammatory diseases by promoting the resolution of inflammation pathways.

Another benefit of using pro-resolution approaches instead of anti-inflammatory approaches is the decreased risk of infection or poor healing. For instance, SPMs have been shown to promote the resolution of inflammation without suppressing antimicrobial function. In fact, SPMs increase neutrophil and macrophage phagocytosis, allowing for the elimination of apoptotic cells and the return of tissue homeostasis (Buckley et al., 2014). Furthermore, macrophages that have phagocytized apoptotic cells will in turn secrete resolvins, maresins, and other SPMs to drive the transition to inflammation resolution (Fullerton, J., Gilroy, D., 2016).

There is an urgent need for additional therapies to prevent PTOA. Resolution of inflammation is understudied in ACL injury and all other joint injuries. The limited existing evidence indicates that interventions aimed at promoting inflammation resolution may be advantageous, emphasizing the necessity for further investigation in this area.

In a recent study conducted by Gowler and colleagues, researchers used a destabilization of the medial meniscus surgical mouse model to induce osteoarthritis. In this study, the researchers found that levels of SPM biosynthetic precursors were negatively associated with pain behaviors and synovitis severity (Gowler, P. R. W. et al., 2022). The role SPMs play in protecting against chronic, low-level inflammation associated with PTOA has yet to be established, but it might be substantial to the finding of therapeutic agents.



**Figure 2: Specialized pro-resolving mediator synthesis pathway:** Summarized biosynthetic pathway leading to the production of SPMs Maresin 1 and Resolvin D1 which bind to receptors LGR6 and FPR2 respectively.

### **Sexual Dimorphism in animal models of PTOA, SPM, and inflammation resolution**

Previous studies that investigated the development of PTOA in mice were conducted using an ACL transection model or a destabilization of the medial meniscus model. The vast majority of these studies were only performed using male mice. Sex is a factor that plays an important role in several diseases, injuries, and healing processes, therefore; it is crucial to consider the sexual dimorphism of PTOA in animal models. Previous research has shown that male mice develop more severe PTOA in comparison to female mice following joint injury (Ma, H.-L. et al., 2007). Female mice seem to have protection from cartilage damage and show different expressions of cytokines in comparison to male mice (Mahr, S. et al., 2003). It is still unknown whether female humans show less severe PTOA than male humans in a clinical setting, however; females are more likely to injure their ACLs in comparison to males (Marmura, H. et al., 2021). Low-level chronic inflammation is heavily associated with the development of PTOA, therefore; the sexual dimorphism between male and female mice in regard to PTOA might be related to inflammation or resolution of inflammation. Previous research comparing levels of specialized pro-resolving mediators in blister exudates between males and females showed that females display a higher concentration of SPMs, especially resolvins, compared to males (Rathod, K. S. et al., 2016). If females display higher activity of SPMs throughout the knee joint following ACL injuries, this may contribute to the decreased severity of PTOA in female mice compared to male mice.

Currently, there is no consensus explaining the biological underpinnings regarding sexual dimorphism in animal models of PTOA, however; investigating the

protection from PTOA that female mice exhibit could help develop therapeutic strategies to mitigate the severity of PTOA.

### **Specific Aims**

Overall, the purpose of this study was to explore the inflammatory response that arises following an ACL injury, which may eventually lead to PTOA. Specifically, the study aimed to examine the potential role that SPMs play in protecting against the development of PTOA. Additionally, the study aimed to determine if there are any sex-based differences in inflammation, resolution of inflammation via SPM production, or the progression to PTOA.

## **METHODS**

### **Mice**

Male and female C57BL6/J mice were obtained at six weeks of age (Jackson Labs, Bar Harbor, ME) and acclimated at the Brigham and Women's vivarium until eight weeks of age. At eight weeks old, which is equivalent to about 18-20 years old in humans, the mice were subjected to ACL transection surgery (ACLT) (Dutta, S. & Sengupta, P. 2016). This age was chosen because the majority of ACL injuries occur in adolescents and young adults who are involved in sports, military training, and other physically demanding activities.

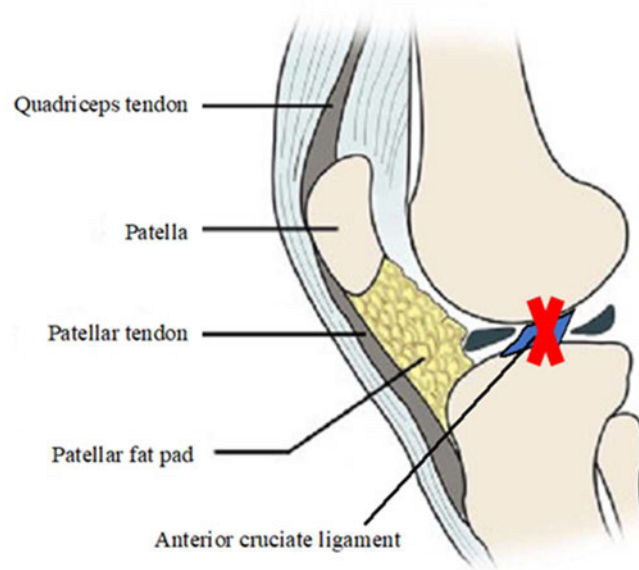
All mice were housed and maintained under Institutional Animal Care and Use Committee (IACUC) regulations at the Brigham and Women's Hospital vivarium under 12-hour light-12-hour dark conditions.

### **Surgery**

For the ACLT, we followed the techniques and guidelines originally explained by Hayami et al. (Hayami et al., 2006). Before the surgery, the mice were anesthetized using an intraperitoneal (I.P.) injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Following anesthetization, eye lube was used on the eyes of the mice to prevent pain and blindness. The operation knees were shaved with an electric razor to remove the fur and allow access to the skin; the unoperated knees were left alone. After shaving, the knees were swabbed with betadine swabsticks and 70% alcohol wipes to create a sterile surface and prevent infection.

To create the initial incision, the skin was tented with tweezers and snipped with scissors. After creating a one-centimeter longitudinal incision over the knee joint, a blade was used to create a parapatellar medial incision in the joint capsule. To expose the ACL, the patella was identified and carefully subluxed laterally. Using a dissecting microscope to visualize the ACL, the fat pad was gently moved to allow for direct access to the ligament. The ACL was then transected with dissecting micro scissors (Figure 3). To confirm the complete transection of the ACL, we examined the presence of the anterior drawer test which confirms the anterior instability of the tibia relative to the femur. The patella was then moved back to its regular anatomical position and sutured into place using absorbable sutures. The skin was also closed with absorbable suturing followed by tissue glue (Medbond). Contralateral, untouched knees were used as controls. The surgery was always performed on the right knees of the mice because “handedness” is not a concept that applies in mice and therefore does not need to be considered. After the mice woke up, we administered buprenorphine as the only postoperative

analgesia. NSAIDs were not used for pain management because research has shown that NSAIDs impair the resolution of inflammation and we aimed to avoid dysregulation in resolutive pathways (Sugimoto et al., 2016).



**Figure 3: Location of ACL transection.** Anatomical representation of the knee joint demonstrating the location of the ACL transection during the surgical procedure.

### Sample Collection

Euthanasia of the mice was performed with primary and secondary methods consisting of carbon dioxide exposure and blood vessel catheterization, respectively. The mice were sacrificed at various time points post-surgery: 1 day, 1 week, 2 weeks, 3 weeks, 4 weeks, and 8 weeks. A total of 3 animals per sex per time point was used.

Synovial fluid was collected by serial lavage of phosphate-buffered saline (PBS). This was done by removing the skin from the knee and creating a small incision over the joint. The serial lavage was done with eight rounds of 2.5  $\mu\text{L}$  of PBS (total of 20  $\mu\text{L}$  per lavage) that was diluted to 100  $\mu\text{L}$  of PBS totalizing 120  $\mu\text{L}$  of lavage fluid. The lavage

fluid was then spun to harvest a pellet of cells and the cells were counted under a microscope using a Neubauer chamber. These cells were then spun in a specialized centrifuge (CytoSpin) to concentrate the synovial cells onto a microscope slide to be stained and examined for the presence of leukocytes. The remaining fluid without cells was collected and frozen for later analysis using ELISA/multiplex.

Periarticular tissues, including synovium and patellar fat pad were stored in RNeasy (Qiagen) to preserve RNA quality, and frozen at  $-80^{\circ}\text{C}$  to later be used for qPCR.

Finally, the remaining tibiofemoral joint consisting of the distal femur and proximal tibia was collected and fixed in freshly prepared paraformaldehyde (PFA) followed by 70% ethanol for further evaluation of micro-computed tomography ( $\mu\text{CT}$ ) and histology. Both the operated and unoperated knees were used for analysis.

### **RNA Extraction**

RNA extraction was performed in the periarticular tissues. To extract the RNA, Trizol was added to Navy bead lysis tubes and the periarticular tissue was added. Tubes were then placed into a Bullet Blender at a speed of 10 for 3 minutes. Tubes were placed in ice to cool down again before repeating the Bullet Blender process again to avoid overheating or rupturing the tubes. The lysate was removed from the tubes and mixed with 20  $\mu\text{L}$  of Proteinase K before being incubated at  $55^{\circ}\text{C}$  for 20 minutes. After this, 110  $\mu\text{L}$  of chloroform was added to each tube before being vortexed for 20 seconds. Tubes were then left at room temperature for 2 minutes before being spun at  $4^{\circ}\text{C}$  and 12000 rpm for 10 minutes. 200  $\mu\text{L}$  of the supernatant was added to 200  $\mu\text{L}$  of 70%

ethanol. The aqueous layer was discarded, and RNA was isolated using the QIAGEN RNeasy kit according to the manufacturer's protocol. Following completion of RNA extraction, the RNA concentration, 260/280, and 260/230 ratios were measured using an ND-1000 NanoDrop Spectrophotometer. RNA was then stored at -80°C to preserve its quality.

### **Real-Time Quantitative Polymerase Chain Reaction (qPCR)**

cDNA was synthesized from isolated RNA (as described above) using the Applied Biosystems High-Capacity cDNA Reverse Transcription Kit according to the manufacturer's protocol. Real-time qPCR was performed using Applied Biosystems Step 20 One Plus Real-Time PCR System with a TaqMan assay. Average cycle threshold (Ct) values were calculated from duplicate technical replicates. Expression of contralateral control knees was set at 1 and the relative expression of each gene at each time point was determined using the delta-delta Ct method (Livak and Schmittgen, 2001).

### **Enzyme-linked Immunosorbent Assay (ELISA)**

To determine the synovial fluid levels of inflammatory cytokines (IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and IL-10) a multiplex assay was performed (Meso Scale Discovery), as previously described (Hunt et al., 2021). Similarly, levels of Mar1 and RvD1 from the synovial fluid were detected by commercially available ELISA kits (Cayman) according to the manufacturer's protocol.

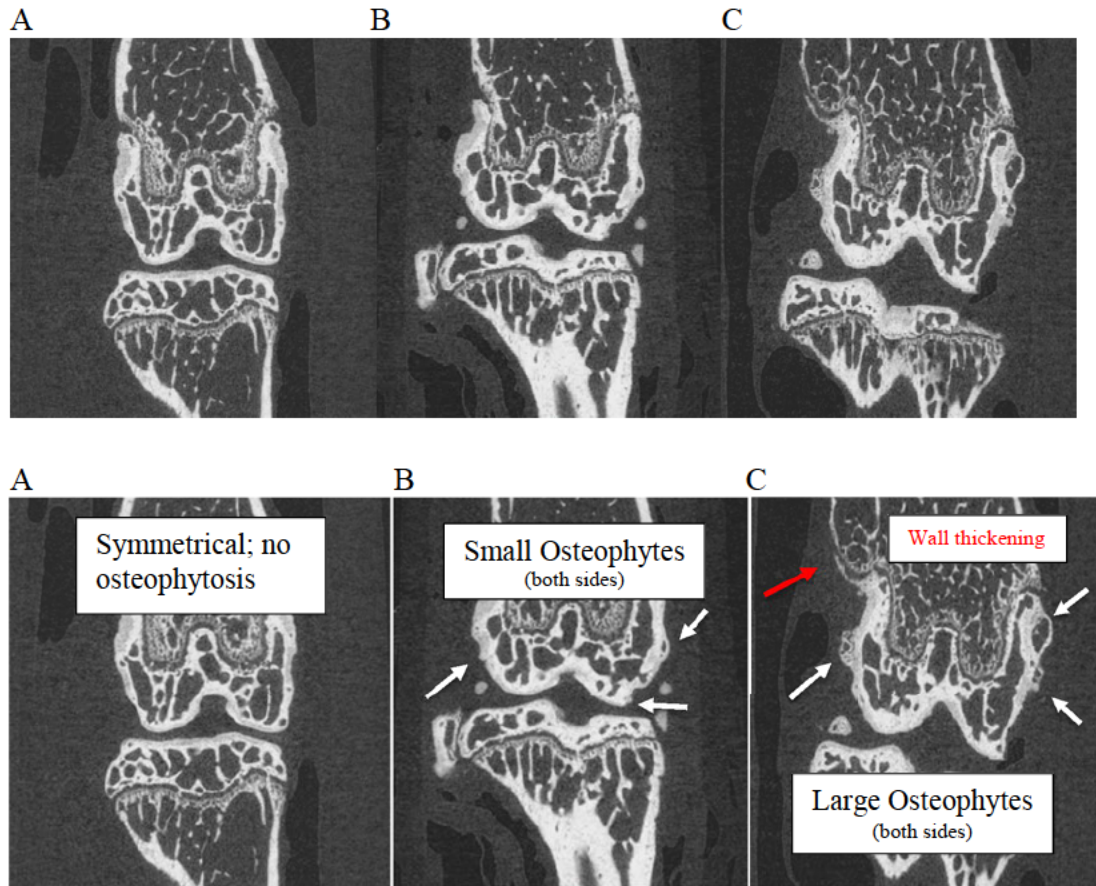
### **Micro-computed tomography ( $\mu$ CT)**

For  $\mu$ CT and histology evaluations, only the 8-week post-ACLT samples were analyzed considering this was the time point when PTOA changes are best observed.

$\mu$ CT scanning of the knee was performed using a Scanco Medical  $\mu$ CT-35 system with a voxel size of 7 $\mu$ m. The bones were scanned in 70% ethanol using an X-ray tube potential, X-ray intensity, and an integration time of 55kVp, 0.145mA, and 600ms respectively. The reference point was set to capture the more distal part of the femur and the proximal part of the tibia. Whole bones were scanned. 3D reconstruction of the entire scan was performed using the manufacturer-supplied software. A radiology software (Horos Project) was used to capture coronal images in four different slices for each bone. The distal femur was scored using a self-made scoring system for asymmetry, abnormal wall thickness, and the presence of osteophytes (Table 1 and Figure 4). This scoring system had an inter-rater reliability of  $\kappa = 0.9$  (data not shown).

	$\mu$ CT Scoring System
0	Normal.
1	Asymmetry between lateral and medial walls of the femur.
2	Thickness of lateral and/or medial walls of the femur.
3	The presence of small osteophytosis in either the lateral or medial wall.
4	The presence of small osteophytosis in both lateral and medial wall.
5	The presence of large osteophytosis in either lateral or medial wall.
6	The presence of large osteophytosis in both lateral and medial wall.

**Table 1: Self-made  $\mu$ CT scoring system.**  $\mu$ CT scoring criteria used to determine the severity of PTOA. Small osteophytes fall in the range of 0.1mm - 0.24mm; large osteophytes  $\geq 0.24$ mm.



**Figure 4:  $\mu$ CT scoring example images.**  $\mu$ CT imaging showing a control knee with a score of 0 (A), an operated knee with a score of 3 (B), and another operated knee with a score of 6 (C).

### Histology

After performing  $\mu$ CT, the tibiofemoral joint was decalcified using 5% Formic acid solution for 5 days. Bones were sent to the Mass General Brigham Histology Core for paraffin embedding and sectioning. Five-micron coronal sections separated by 45 $\mu$ m were obtained spanning the entire tibiofemoral joint.

Prior to staining, slides were heated at 60°C overnight to melt the paraffin and cooled at room temperature for 30 minutes. Slides were then deparaffinized by being submerged in histoclear followed by 100% ethanol, 95% ethanol, and finally 70% ethanol. We used a Safranin O and Fast Green Stain to best visualize the cartilage, and therefore, the degree of post-traumatic osteoarthritis. This stain was obtained by first staining with Weigert's Iron Hematoxylin followed by Fast Green and finally, Safranin O. PTOA severity was assessed by two independent, blinded examiners using OARSI histopathology scoring (Table 2).

Grade	Osteoarthritic damage
0	Normal
0.5	Loss of Safranin-O without structural changes
1	Small fibrillations without loss of cartilage
2	Vertical clefts down to the layer immediately below the superficial layer and some loss of surface lamina
3	Vertical clefts/erosion to the calcified cartilage extending to <25% of the articular cartilage
4	Vertical clefts/erosion to the calcified cartilage extending to 25-50% of the articular cartilage
5	Vertical clefts/erosion to the calcified cartilage extending to 50-75% of the articular cartilage

6	Vertical clefts/erosion to the calcified cartilage extending >75% of the articular cartilage
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**Table 2: OARSI scoring system for histological samples.** OARSI scoring criteria to determine the severity of cartilage damage using Safranin O-stained samples.

### LGR6 KO Mice

LGR6 is a known receptor for the SPM Maresin 1 (MaR1). To further explore the role of MaR1 in PTOA development, *Lgr6* knockout (KO) male and female mice as well as wild-type (WT) male and female mice from the same lineage underwent ACLT using the same technique. After 8 weeks post-surgery, animals were euthanized, and tibiofemoral joint samples were collected for analyses of microCT and histology. Samples were scored according to the same score systems previously described.

### Statistical Analysis

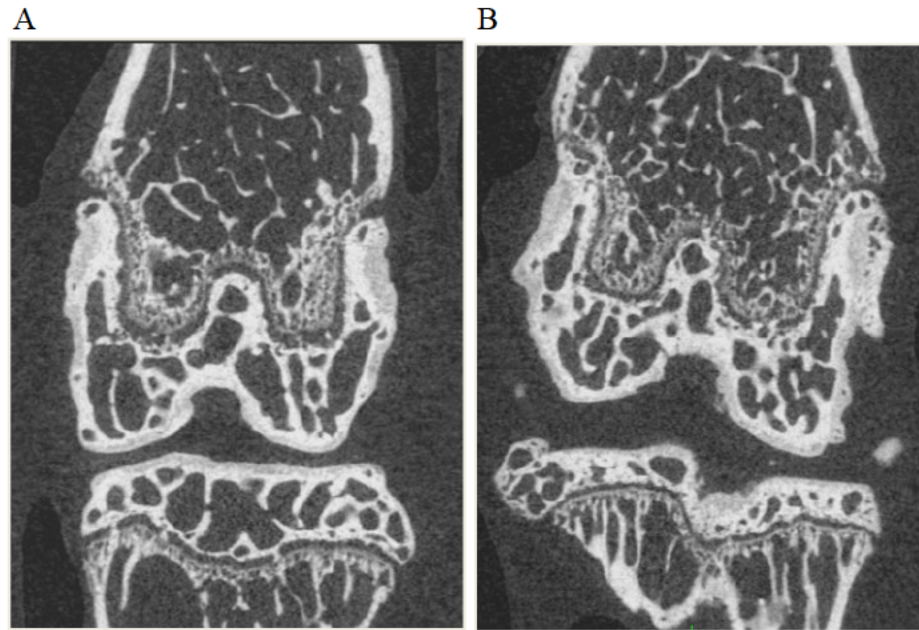
Statistical analysis and graphing were performed using GraphPad Prism 9. Unpaired parametric Student's t-tests were used for comparing two groups and ANOVA tests were used for comparing greater than two groups. Data was assumed to be normally distributed. P values less than 0.05 were considered statistically significant.

## RESULTS

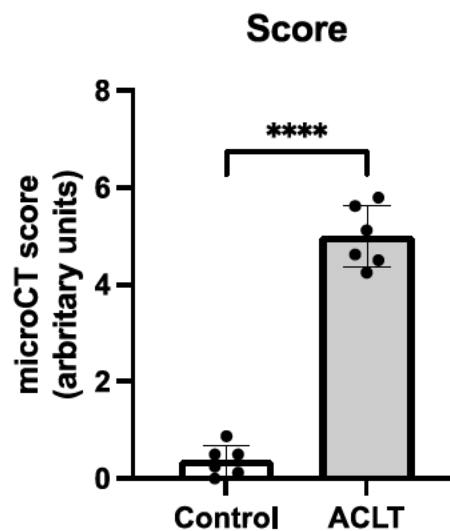
### ACL Transection Leads to PTOA Development

$\mu$ CT imaging confirmed that our surgical mouse model leads to the development of PTOA. Using the scoring system mentioned previously (Table 1), we found that ACL injury leads to deformity of the affected joint (Fig. 5). There was statistical significance in the  $\mu$ CT score between the control and operated knees, with the operated knees having a higher overall score (Fig. 6).

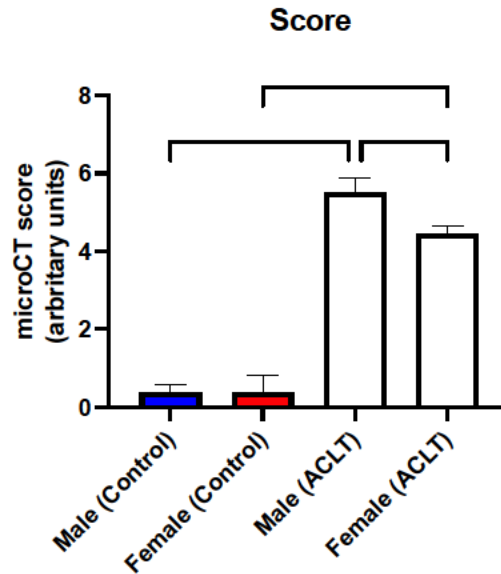
Through analysis of the  $\mu$ CT images we were also observed that, on average, female mice showed less severe signs of PTOA in comparison to male mice, confirming our hypothesis that females possess some protective mechanisms against PTOA (Fig. 7).



**Figure 5:  $\mu$ CT imaging shows signs of PTOA in ACLT knees.** A control, unoperated knee with a  $\mu$ CT score of 0 (A) and an operated, ACLT knee with a score of 6 (B) 8 weeks post-surgery.



**Figure 6:  $\mu$ CT shows ACLT significantly increases the signs of PTOA in mice.**  $\mu$ CT analysis and scoring revealed that ACLT knees had a significantly higher  $\mu$ CT score (more severe signs of PTOA) in comparison to control, unoperated knees. \*\*\*\*,  $p < 0.0001$ , one way ANOVA with Tukey multiple comparison post-test.

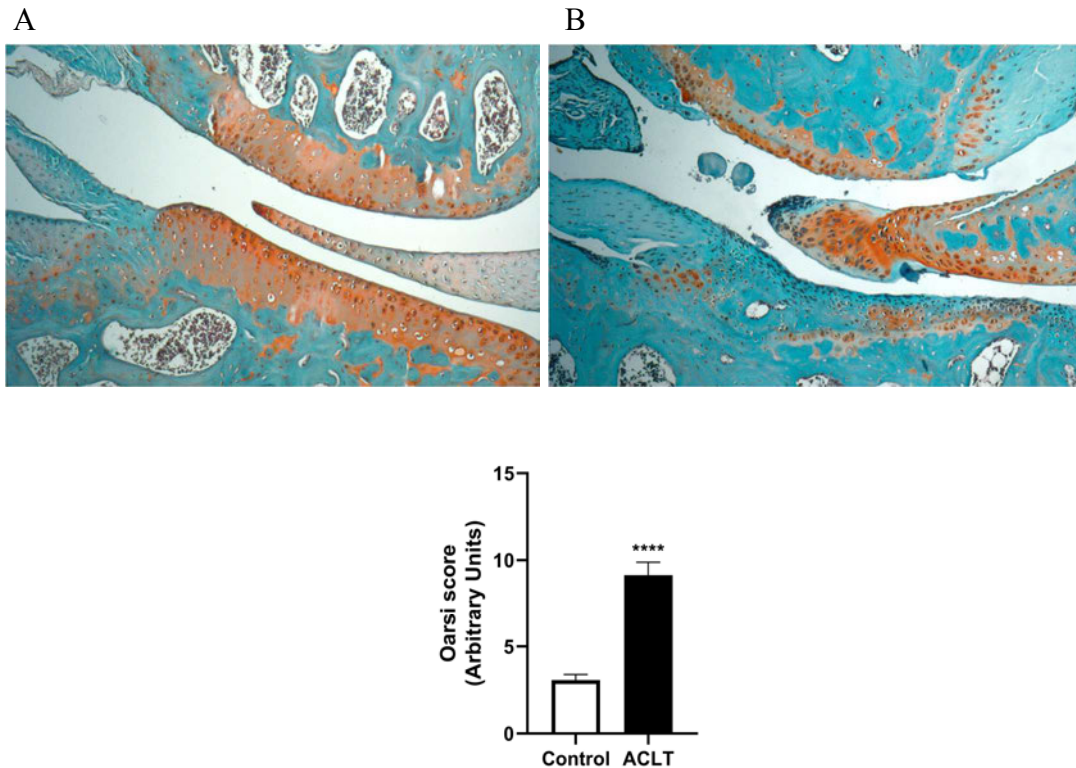


**Figure 7:  $\mu$ CT shows that PTOA severity after ACLT is more severe in males than females.** The  $\mu$ CT scoring system show that male mice have a significantly higher score (more severe signs of PTOA) in comparison to female mice. \*,  $p < 0.05$ , \*\*\*,  $p < 0.001$ , \*\*\*\*,  $p < 0.0001$ , two-way ANOVA with Tukey multiple comparison post-test.

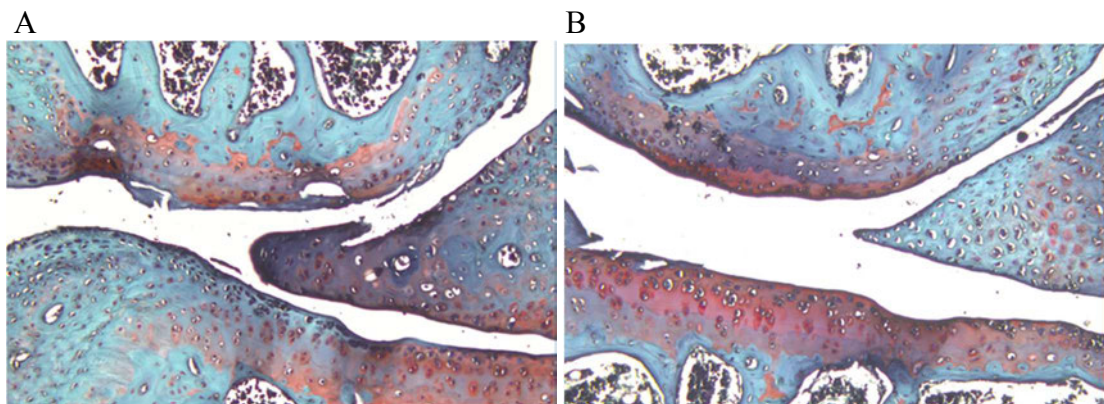
Similar to the  $\mu$ CT analysis, histology analysis using the OARSI score showed that ACL transected knees showed a development of PTOA in comparison to control knees (Fig. 8).

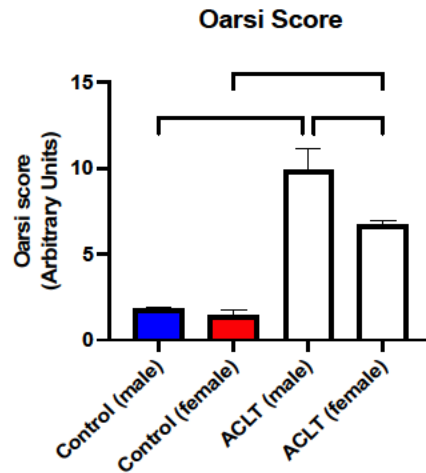
Through histology images, we saw that ACLT knees stained with Safranin-O showed a loss of proteoglycans (reduction in the orange stain) as well as cartilage irregularities (Fig. 8). The idea that the endogenous production of SPMs is not enough to prevent PTOA was made evident by both the  $\mu$ CT and histology data.

Once again, data was separated by sex and evaluated for potential differences in scoring. The histology data confirmed our earlier findings that males show more severe signs of PTOA (Fig. 9).



**Figure 8: Histology shows ACLT significantly increases the signs of PTOA in mice.** Histological samples stained with Safranin-O shows that ACLT mice (B) had superior OARSI score (more severe signs of PTOA) in comparison to control mice (A). \*\*\*\*,  $p < 0.0001$ , one way ANOVA with Tukey multiple comparison post-test.





**Figure 9: Histology shows that PTOA severity after ACLT is more severe in males than females.** Safranin O/fast green stained sections spanning the tibiofemoral joint were evaluated by two independent observers using OARSI scoring criteria. Representative images from males (A) and females (B) are shown and average OARSI scores for each group (n=3 for males, n=3 for females) is graphed (C). \*,  $p < 0.05$ , \*\*,  $p < 0.01$ , \*\*\*\*,  $p < 0.0001$ , one way ANOVA with Tukey multiple comparison post-test.

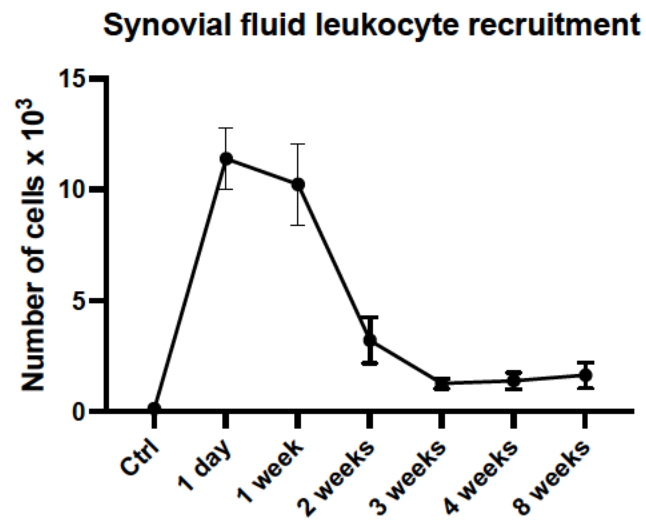
### Inflammation

Cell counting showed that synovial fluid leukocyte recruitment peaked right after the ACLT, between one day to one-week post-surgery (Fig. 10). Although this measure of inflammation began to decrease at two weeks post-ACLT, levels above control persisted at 8 weeks after surgery. This suggests the presence of chronic inflammation in the form of leukocyte recruitment following ACL injury.

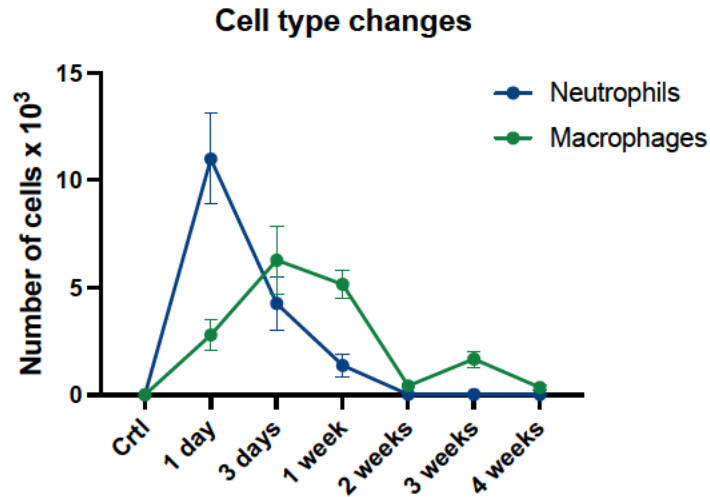
In terms of leukocyte cells, our model followed a typical inflammatory trajectory with an increase in neutrophil recruitment at day one post-surgery, followed by a subsequent increase in macrophages around day three and onward (Fig. 11).

When the data was separated by sex, the overall cell count was very similar between male and female mice, except on day 3 when female mice appear to have less leukocytes in the synovial fluid (Fig. 12).

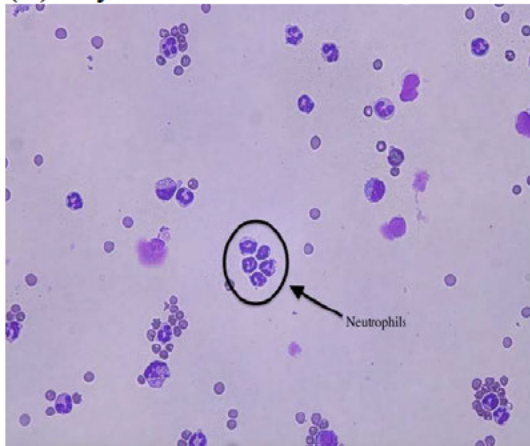
In regard to cell types, neutrophils were similar between the sex, however the macrophage count was higher at day 3 in males than in females (Fig. 13).



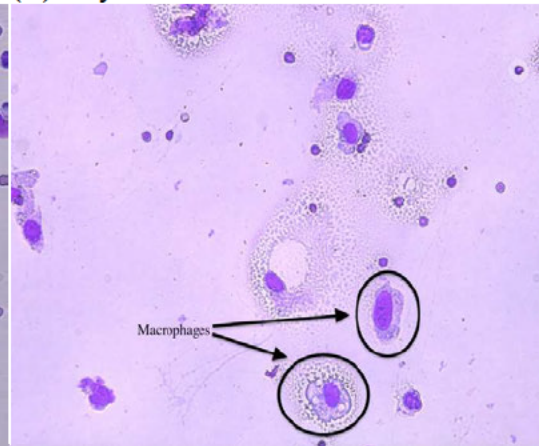
**Figure 10: Leukocyte recruitment peaked between one day to one-week post-ACLT.** Cell counting showed that nucleated cells in joint lavage peak at 1 day-1 week after ACLT surgery, but never return to control levels.



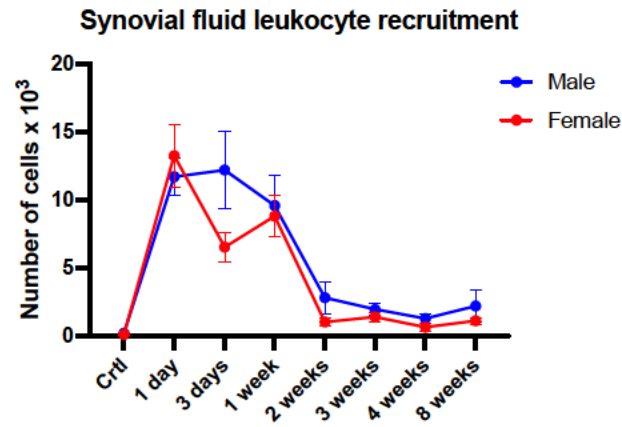
(A) Day 1



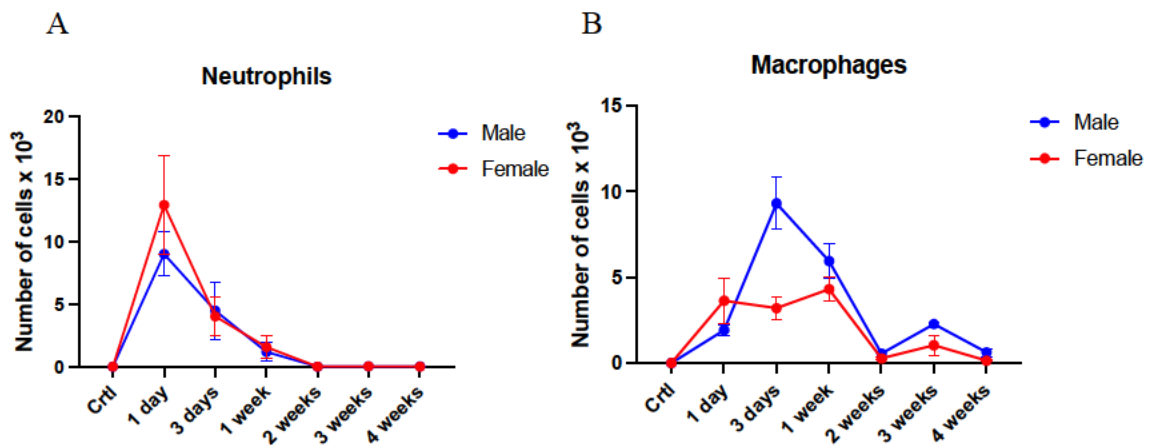
(B) Day 3



**Figure 11: Neutrophil recruitment to the joint precedes macrophages.** Cells were transferred onto a microscope slide using the CytoSpin centrifuge to examine the types of leukocytes. Initially, there is an increase in neutrophils at day 1 (A) post-surgery, followed by an increase in macrophages at day 3 (B).



**Figure 12: Female mice have less leukocytes than males at 3 days post-ACLT.** Manual cell count was similar between the sexes, except on day 3 when female mice show significantly less leukocytes in the synovial fluid compared to male mice ( $p < 0.01$ ). Two-way ANOVA with Tukey multiple comparison post-test.



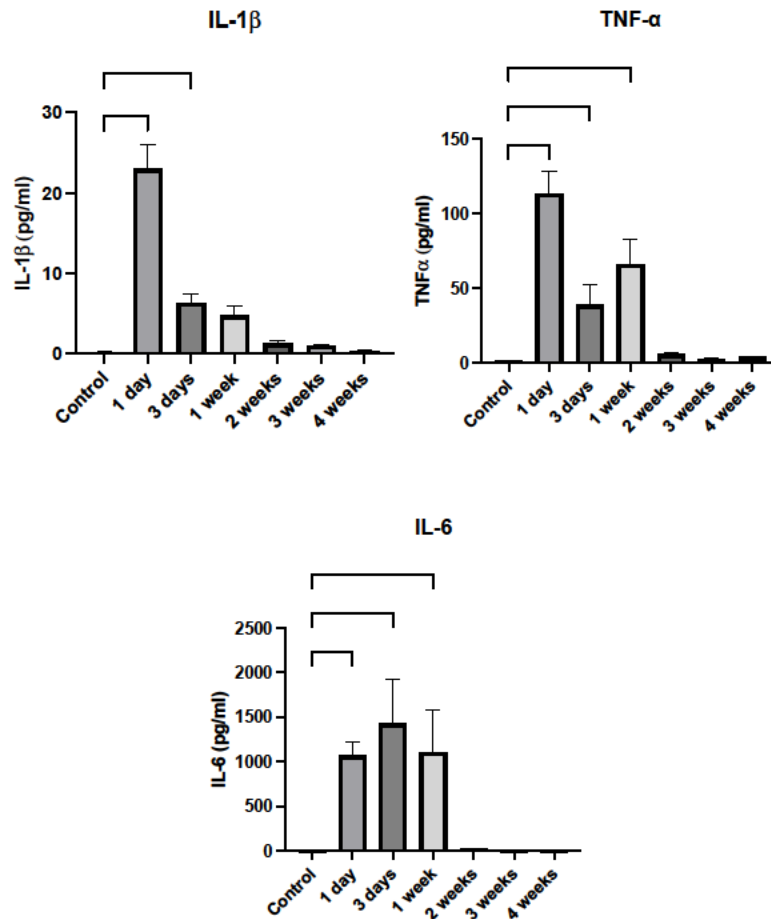
**Figure 13: Macrophage concentration was higher in males compared to females at 3 days post-ACLT.** Manual cell counting showed that neutrophils were similar between the sexes (A); however, the macrophage count was significantly higher at day 3 post-surgery in males compared to females (B) ( $p < 0.0001$ ). Two-way ANOVA with Tukey multiple comparison post-test.

Multiplex data was collected using the synovial fluid to measure the levels of inflammatory cytokines: IL-1  $\beta$ , TNF-  $\alpha$ , IL-6, and IL-10. Data showed that the ACLT led to the release of pro-inflammatory cytokines right after surgery (Fig. 14). ACLT also

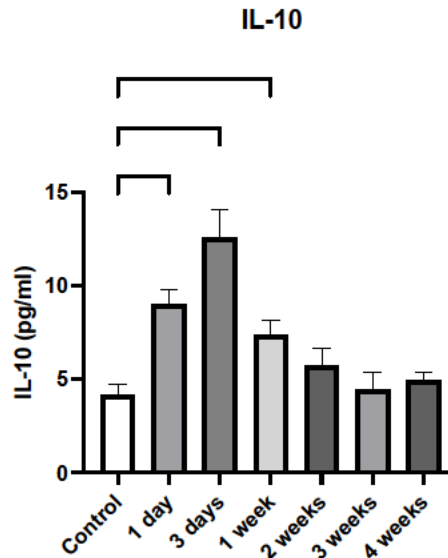
led to anti-inflammatory IL-10 cytokine production, which peaked slightly later than the inflammatory cytokine peak (Fig. 15). No significant sex differences in inflammatory cytokine levels were observed (data not shown).

Chemokines CXCL1 and CCL2 were also measured with a multiplex assay.

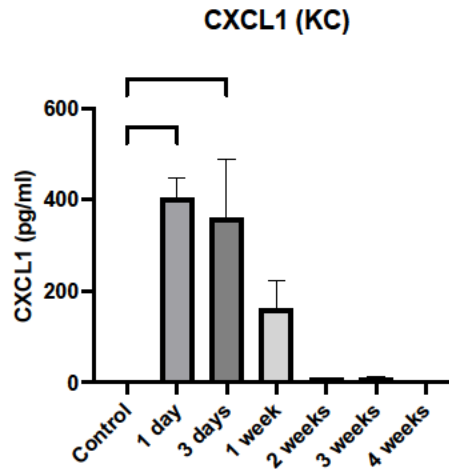
CXCL1 is a chemokine for neutrophil attraction and CCL2 (also known as MCP-1) is for macrophage recruitment. Both CXCL1 and CCL2 showed the greatest peak at one day post-surgery (Fig. 16 and 17). We then asked if there were sex differences in the expression of these two chemokines. Both CXCL1, and more remarkably, CCL2 were much higher in female mice one day after surgery compared to male mice (Fig. 18).



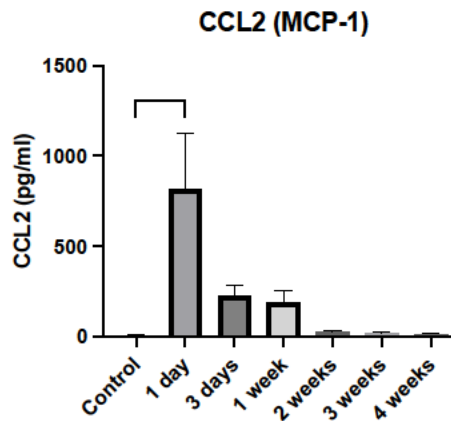
**Figure 14: IL-1  $\beta$ , TNF-  $\alpha$ , and IL-6 generally show the greatest peak in production directly after ACLT.** Multiplex ELISA data was collected using the synovial fluid to measure the levels of inflammatory cytokines IL-1  $\beta$ , TNF-  $\alpha$ , and IL-6. These cytokines show a significant increase in concentration approximately 1 day to 1-week post-ACLT in comparison to baseline levels. \*,  $p < 0.05$ , \*\*,  $p < 0.01$ , \*\*\*,  $p < 0.001$ , \*\*\*\*,  $p < 0.0001$ , one way ANOVA with Tukey multiple comparison post-test.



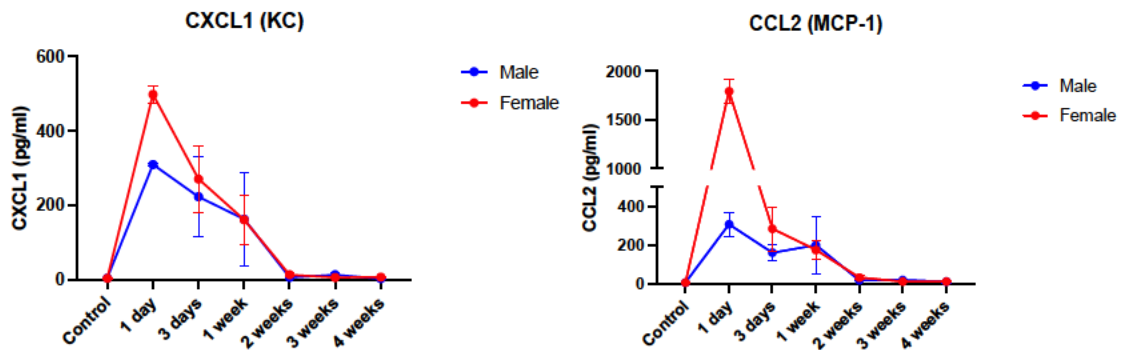
**Figure 15: IL-10 shows the greatest peak in concentration 3 days post ACLT.** Multiplex ELISA data was collected using the synovial fluid to measure the levels of anti-inflammatory cytokine IL-10. IL-10 shows a significant increase in concentration from 1 day to 1-week post-surgery in comparison to baseline levels, with the greatest peak at 3 days. \*,  $p < 0.05$ , \*\*,  $p < 0.01$ , \*\*\*\*,  $p < 0.0001$ , one way ANOVA with Tukey multiple comparison post-test.



**Figure 16: CXCL1 shows the greatest peak in concentration 1-3 days post-surgery.** Multiplex ELISA was used to measure levels of CXCL1 in synovial fluid. Results showed that CXCL1 concentration was significantly higher at 1-3 days post-surgery in comparison to baseline levels. \*\*\*,  $p < 0.001$ , \*\*\*\*,  $p < 0.0001$ , one way ANOVA with Tukey multiple comparison post-test.



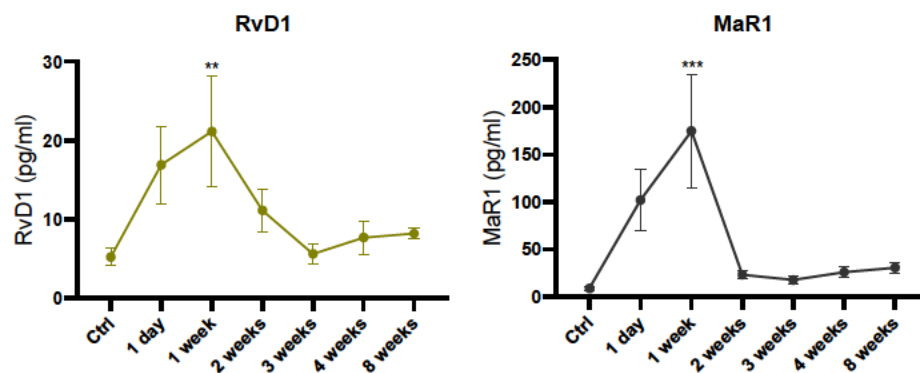
**Figure 17: CCL2 shows the greatest peak in concentration one day post-surgery.** Multiplex ELISA was used to measure levels of CCL2 in synovial fluid. Results showed that CCL2 concentration was significantly higher at 1 day post-surgery in comparison to baseline levels. \*\*\*\*,  $p < 0.0001$ , one way ANOVA with Tukey multiple comparison post-test.



**Figure 18: CXCL1 and CCL2 were higher in females than males 1 day after surgery.** Multiplex ELISA was used to measure levels of CXCL1 and CCL2 in synovial fluid. Results showed that both were higher in female mice compared to male mice; however, only the sex difference at day 1 with CCL2 showed statistical significance.  $p < 0.001$ , two way ANOVA with Tukey multiple comparison post-test.

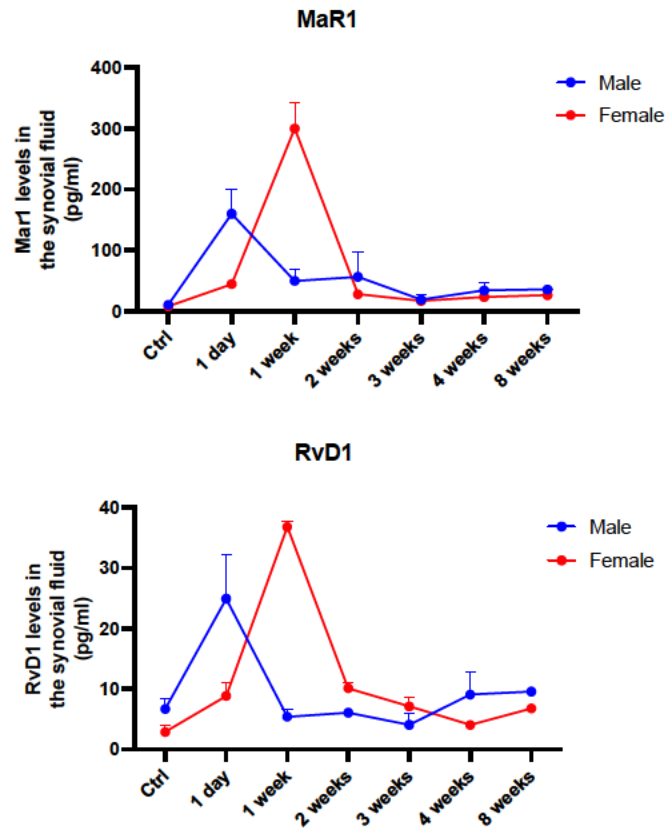
### Resolution of Inflammation

Levels of endogenous SPMs Mar1 and RvD1 were measured by ELISA. Data showed that the ACLT surgery led to a peak in SPM production at one week post injury (Fig. 19). When separating the data by sex, male mice had an earlier and inferior peak of Mar1 and RvD1 production, while females had higher and later production (Fig. 20).



**Figure 19: ACLT leads to MaR1 and RvD1 production, mostly 1 week post-surgery.** ELISA was used to measure levels of SPMs Mar1 and RvD1 in synovial fluid. Results showed that the concentrations of both SPMs increased significantly at one week post-

surgery. \*\*,  $p < 0.01$ , \*\*\*,  $p < 0.001$ , One way ANOVA with Tukey multiple comparison post-test.



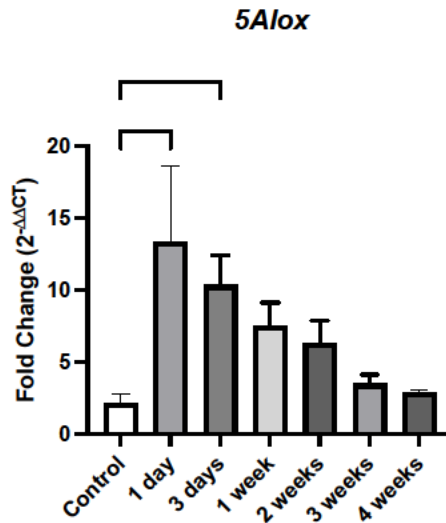
**Figure 20: Male mice had a smaller, earlier peak of MaR1 and RvD1.** ELISA was used to measure levels of SPMs MaR1 and RvD1 in synovial fluid. Results showed that male mice has a smaller, earlier peak in both SPMs compared to female mice. MaR1 and RvD1 were significantly higher in females compared to males at one week post-surgery.  $p < 0.001$ , two way ANOVA with Tukey multiple comparison post-test.

As mentioned earlier, qPCR was performed with the collected periarticular tissue using the TaqMan technique. We were interested in measuring the presence of SPM biosynthetic enzymes after the ACLT. 5Alox and 12/15Alox were the specific enzymes measured; 5Alox is a key enzyme in the synthesis of pro-inflammatory leukotrienes whereas 12/15Alox is more specific to SPM production. The qPCR results showed that

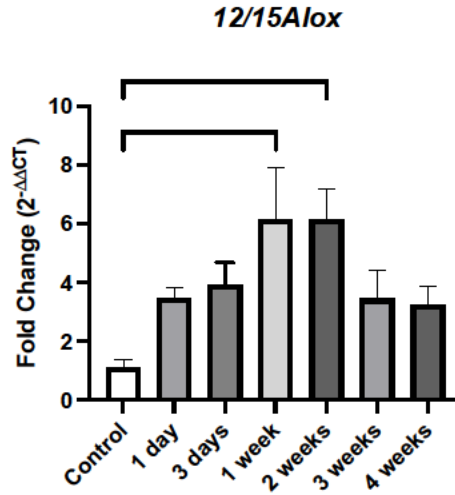
5Alox peaked at one day post-surgery while 12/15Alox peaked at one-week post-surgery (Fig. 21 and 22). This difference in expression is probably due to the early peak of pro-inflammatory leukotrienes.

When examining the sex differences, we found that male mice show a higher expression of 5Alox one day post-ACLT in comparison to female mice (Fig. 23).

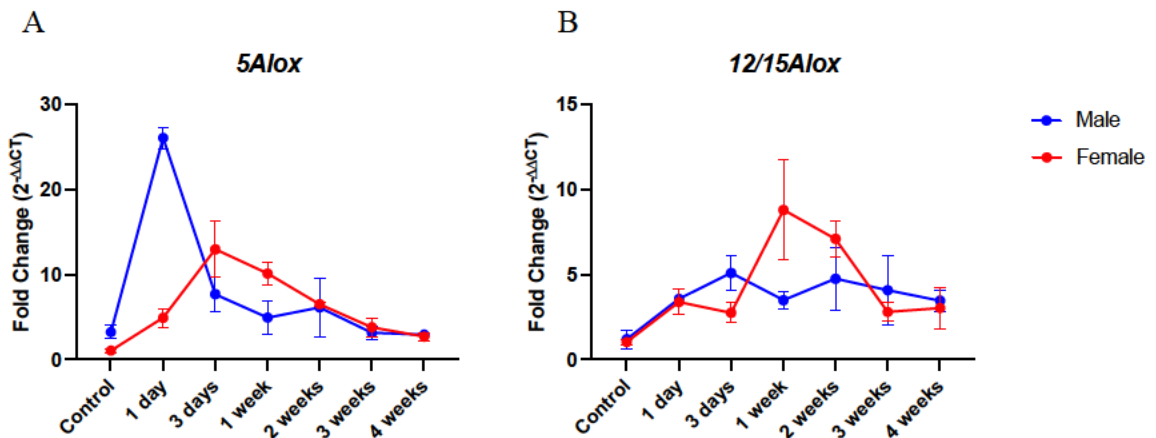
Furthermore, the expression of 12/15Alox showed a higher expression in females 1-2 weeks post-surgery (Fig. 23).



**Figure 21: 5Alox shows greatest peak in expression one day post ACLT.** qPCR was performed to examine the expression of Biosynthetic enzyme 5Alox. Data shows that the greatest peak in expression occurs one day post ACLT compared to baseline levels. \*,  $p < 0.05$ , \*\*,  $p < 0.01$ , one way ANOVA with Tukey multiple comparison post-test.



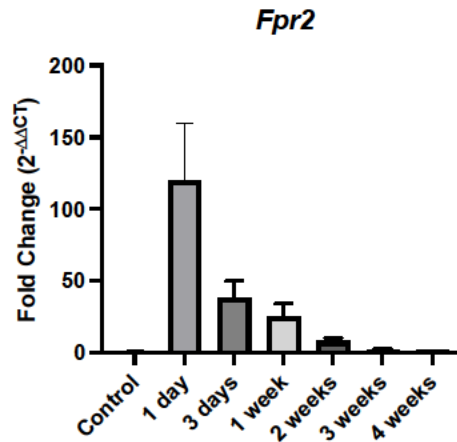
**Figure 22: 12/15Alox shows greatest peak in expression one to two weeks post ACLT.** qPCR was performed to examine the expression of Biosynthetic enzyme 12/15Alox. Data shows that the greatest peak in expression occurs one to two weeks post ACLT compared to baseline levels. \*\*,  $p < 0.01$ , \*\*,  $p < 0.01$ , one way ANOVA with Tukey multiple comparison post-test.



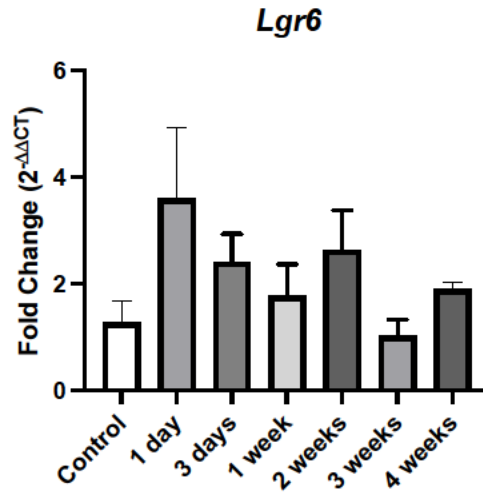
**Figure 23: Expression of biosynthetic enzymes 5Alox and 12/15Alox.** qPCR data showed that male mice show a significantly higher expression of 5Alox one day post-ACLT in comparison to female mice (A) ( $p < 0.0001$ ). The enzyme 12/15Alox showed a higher expression in females 1-2 weeks post-surgery; however, it was not statistically significant (B). Two-way ANOVA with Tukey multiple comparison post-test.

We also examined the expression of LGR6, a receptor for Mar1, and FPR2, which is a receptor for RvD1 but also a receptor for other resolutive molecules and pro-inflammatory molecules. Data showed that the ACLT increased the expression of both SPM receptors in the periarticular tissue (Fig. 24 and 25).

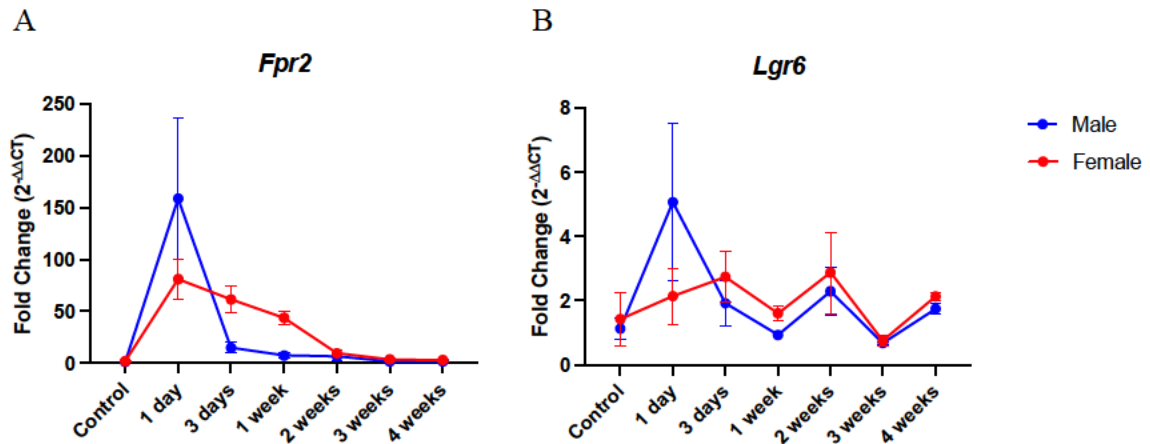
When separating the data by sex, male mice appear to have a higher expression of FPR2 and Lgr6 in comparison to female mice at one day post-surgery (Fig. 26).



**Figure 24: Expression of FPR2 after surgery.** qPCR was performed to examine the expression of RvD1 receptor FPR2. This receptor shows the greatest peak in expression at one day post ACLT compared to baseline levels.  $p < 0.001$ , one way ANOVA and Tukey multiple comparison test.



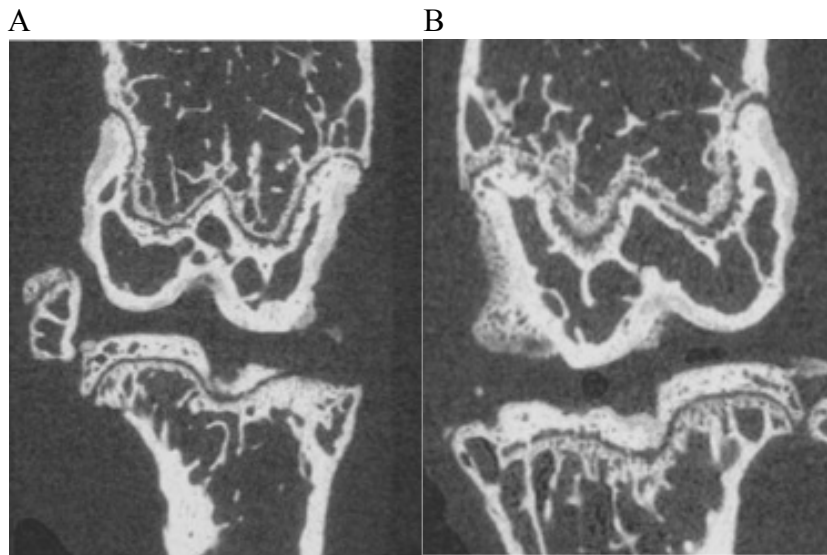
**Figure 25: Expression of LGR6 after surgery.** qPCR was performed to examine the expression of Mar1 receptor LGR6. This receptor seems to show the greatest peak in expression one day post ACLT; however, it is not statistically significant. One way ANOVA with Tukey multiple comparison post-test.

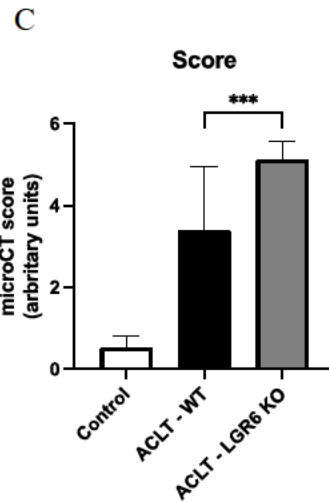


**Figure 26: Expression of receptors FPR2 and LGR6.** qPCR data showed that male mice appear to have a higher expression of FPR2 and Lgr6 in comparison to female mice at one day post-surgery; however, this trend is not significant and requires a larger sample number to be confirmed. Two-way ANOVA with Tukey multiple comparison post-test.

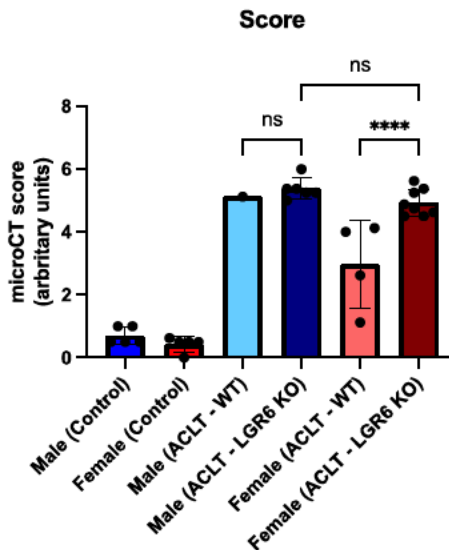
The key test of the importance of SPMs was performing the ACLT surgery on mice deficient in the receptor for SPM Mar1, LGR6. We analyzed  $\mu$ CT data in mice who had the LGR6 deficiency mutation. When the data from both sexes are combined, the operated knees from mice with the LGR6 deficiency showed more severe signs of PTOA in comparison to wild-type (WT) mice (Fig. 27).

Interestingly, when the LGR6 data was separated by sex, we saw that the statistical significance in PTOA severity between WT and LGR6 KO mice only appeared in female mice, meaning female LGR6 KO had worse signs of PTOA compared to female WT mice (Fig. 28). This data is still preliminary as our study currently only includes one WT male mouse. Our conclusion requires a larger sample size for confirmation.





**Figure 27: PTOA severity after ACLT is more severe in mice with LGR6 KO mutation.** When wild-type mice knees (A) are compared to LGR6 KO mice (B) using the  $\mu$ CT scoring system, the LGR6 KO mice show significantly more severe signs of PTOA (C). \*\*,  $p < 0.001$ , one way ANOVA with Tukey multiple comparison post-test.



**Figure 28: Increased PTOA severity in LGR6 KO mice may be specific to female mice.** In females, PTOA severity based on  $\mu$ CT score was significantly worse in LGR6KO mice (\*\*\*\*,  $p < 0.0001$ ), while no significant difference was observed in a

smaller cohort of male mice. Two-way ANOVA with Tukey multiple comparison post-test.

## **DISCUSSION**

With this study, we found that our ACLT mouse model was able to consistently demonstrate reliable progressive cartilage degradation and other symptoms of PTOA in both male and female mice. This finding was consistent with previous research using a similar ACLT model to induce OA (Hayami et al., 2012). Our ACLT model also allowed us to observe the stepwise progression of symptoms from injury to PTOA including the initial acute inflammation, chronic low-grade synovitis, and chondral breakdown of articular cartilage surfaces.

The first step in the progression towards PTOA was the initial acute inflammation following the ACLT. This agrees with previous investigations that shows that ACL injury leads to an inflammatory response, with recruitment of immune cells and release of cytokines and chemokines (Lattermann et al., 2018). We found that the presence of inflammatory cells decreased at later time points, implying the presence of inflammation resolution during this process. It could be possible that inflammatory molecules at later time points might include M2 macrophages after the switch from M1 macrophages; however, flow cytometry data would need to be collected to determine the phenotypes of these cells. We are currently attempting to create a sufficient method to collect cells from the synovial tissue for flow cytometry testing. There have been several studies that show that SPMs can switch macrophages from the M1 to the M2 phenotype. A paper by Zhang et al. investigated the effect of resolvin D2, a type of SPM, on postischemic revascularization and resolution of inflammation. In this study, the researchers found that

resolvin D2 treatment led to a shift in macrophage polarization towards the anti-inflammatory M2 phenotype (Zhang et al., 2016). In another study, researchers investigated the effects of SPMs maresin 1 and resolvin D2 on atheroprotection in mice. In this study, the authors found that treatment with these SPMs decreased plaque size and increased the proportion of M2-like macrophages. They also found that the SPMs decreased the expression of pro-inflammatory cytokines and increased the expression of anti-inflammatory cytokines. Overall, the researchers suggest that these SPMs have a potential therapeutic effect through their regulation of macrophage polarization (Viola et al., 2016).

Previous studies have confirmed the claim that 12/15Alox is an important biosynthetic enzyme for inflammation resolution (Kronke et al., 2009). A study by Kronke et al. found that mice without the 12/15Alox enzyme show a greater severity of inflammation and tissue damage (Kronke et al., 2009). With our qPCR analysis of 5Alox, an SPM biosynthetic enzyme that is also related to pro-inflammatory leukotrienes, and 12/15Alox, a biosynthetic enzyme specific to SPM production, we found an increase in 5Alox in males compared to females one day after surgery and an increase in 12/15Alox in females compared to males one week after surgery (Werz, 2002; Serhan et al., 2015). The 5Alox data could suggest that males experience a larger induction of pro-inflammatory leukotrienes compared to females. This finding might be one of the reasons that male mice appeared to have more severe PTOA than females in our study. The 12/15Alox data might suggest greater SPM production in female mice than male mice at one-week post-surgery. If this is true, an increased production of SPMs would help aid in

the resolution of inflammation and might be a factor as to why females experience less severe PTOA in this ACLT mouse model. As stated earlier, further testing to determine macrophage phenotypes via flow cytometry is necessary.

qPCR data also showed that ACLT induced an increase in the expression of SPM receptors LGR6 and FPR2. Furthermore, the induction and presence of these receptors support the idea that SPMs can actually exert their function.

Multiplex ELISA showed that female mice show an increased expression of CCL2, a chemokine responsible for macrophage recruitment, compared to male mice one day post-surgery. Given the female mice's protection against severe PTOA compared to male mice, this higher level of CCL2 in females might be related to the early recruitment of macrophages which is essential for proper tissue repair (Wynn and Vannella, 2016). Authors of a review article note that early recruitment of macrophages to injured tissue is crucial for tissue repair and regeneration, and that macrophages play a critical part in clearing debris, promoting angiogenesis, and aiding in collagen deposition (Wynn and Vannella, 2016). Interestingly, there have been several studies that suggest that females may have a higher induction of M2 macrophages compared to males. For example, in a study by Liao et al., the researchers investigated the role of Krüppel-like factor 4 (KLF4) in macrophage polarization. The researchers found that KLF4 is required for the polarization of macrophages from the M1 to the M2 phenotype, as silencing KLF4 led to decreased expression of M2 macrophages and increased expression of M1 macrophages. Interestingly, they also observed sex differences in KLF4 expression, with female

macrophages expressing higher levels of KLF4 than males, which they believe contributed to a higher induction of M2 macrophages in females (Liao et al., 2011).

ELISA measuring the levels of endogenous SPMs showed that female mice showed a higher and later peak production of Mar1 and RvD1 compared to male mice. Considering SPMs are an integral part of the resolution of inflammation pathway, this difference may influence the better outcomes of PTOA found in females. A study by Ma et al. found that male mice display more severe PTOA following a destabilization of the medial meniscus compared to female mice (Ma et al., 2007). Their results were attributed to the difference in sex hormones in males versus females; however, the relationship between sex hormones and SPM production would be interesting to investigate.

Our study demonstrates that SPMs important for inflammation resolution, MaR1 and Rvd1, increase in the joint in response to ACL injury. Other studies have also found the presence of SPMs in the joint. For example, in a study by Serhan and colleagues the researchers measured the levels of SPM resolvin E1 and found that it was present in the synovial fluid of mice with inflammatory arthritis. They also observed that the expression of enzymes involved in SPM biosynthesis, such as 5-lipoxygenase and 15-lipoxygenase, were increased in the joint tissues of arthritic mice, suggesting that SPM production may be upregulated during joint inflammation. (Serhan et al., 2008). However, endogenous pro-resolving pathways incompletely resolve inflammation in our model, as demonstrated by elevated synovial cell count at 8 weeks post-ACLT. Chronic inflammation after ACL transection surgery leads to PTOA as quantified by  $\mu$ CT imaging and histology analysis at 8 weeks post-surgery. This implies that the mechanisms in place attempting to resolve

inflammation, such as physiologically produced SPMs, do not completely resolve the inflammation that leads to PTOA. We hypothesize that supplementation of SPMs after injury could improve inflammation resolution and PTOA outcomes and will test this in future work.

Through  $\mu$ CT and histology, we also observed that female mice tend to show less severe signs of PTOA than male mice. This suggests that female mice have some protective mechanisms, which could relate to the efficiency in their resolution of inflammation. Previous research has also found that females show less severe osteoarthritis following injury in comparison to males. A study by Mahr et al. found that female mice were better protected from cartilage degradation in comparison to male mice due to increased expression of TGF $\beta$ 1 and IL4 (Mahr et al., 2003). The idea that females possess some protective mechanisms against arthritis seems consistent amongst the literature and our current study; however, the exact reason behind this protection needs to be further considered.

We also hypothesized that SPM deficiency would worsen PTOA that follows an ACL injury. We tested this hypothesis with LGR6 KO mice that lacked the receptor for the SPM Mar1.  $\mu$ CT data showed that mice with the LGR6 KO mutation showed more severe signs of PTOA than WT mice. This conclusion is currently being further verified through histologic analysis with OARSI scoring. This suggests that when SPM Mar1 is unable to properly bind to its receptor (LGR6), the result is a more severe form of PTOA, meaning that Mar1 might play a crucial role in mitigating the severity of PTOA. If Mar1 is important for the resolution of inflammation following ACL injuries, then perhaps the

administration of exogenous Mar1 would help alleviate the severity of PTOA following ACLT.

$\mu$ CT data also showed that female mice were more negatively affected by the LGR6 KO mutation than male mice; however, a larger male sample size is needed to confirm this data's significance. This suggests that the SPM Mar1 could play a more important, potent role in female mice compared to male mice.

In the field of orthopedics research, scoring techniques for assessing arthritis severity are crucial for accurately evaluating experimental outcomes. While previous studies have utilized various scoring methods for analyzing  $\mu$ CT data in PTOA models, such as calculating the percent bone volume (bone volume/trabecular volume), trabecular thickness, and bone surface/bone volume, we wanted to focus more on the formation of osteophytes and joint deformity (Kim and Kang, 2015). Thus, we created a new scoring mechanism that was simple yet effective and had a high interclass correlation coefficient (ICC), ensuring the reliability and reproducibility of our results (data not shown). This allowed us to accurately and objectively evaluate the severity of arthritis in our animal model and will eventually help us better understand the effectiveness of potential therapeutic interventions.

### **Limitations**

Although we were able to successfully replicate the development of PTOA following ACL injury in our surgical mouse model, there are a few limitations to acknowledge. First and foremost, the entirety of our data was collected on mice. While mice are a great model organism, it cannot be said whether our results would be

replicated in a human study. In order to gain the information necessary to benefit human patients, similar data must be collected on human subjects.

Furthermore, our method for causing the ACL injury was surgical. The surgical ACL transection allowed us to induce consistent ACL injuries and knee traumas among the mice in a controlled setting; however, in patient scenarios, ACL injuries occur due to variable complex movements. Moreover, many human patients choose to undergo ACL reconstruction surgery to permanently repair the ligament and restore knee stability and function and assessing inflammatory and pro-resolving pathways after reconstruction would be important. However, our ability to model this in mice is limited as we are unable to perform ACL reconstruction surgeries due to the microscopic size of the mice's ligaments.

A significant limitation in interpreting our data is the choice of unoperated contralateral knee as a control. A better control would be a sham surgery. A sham surgery is an exact replica of the ACLT surgery without cutting the ligament. This data is important to prove that the chronic inflammation and PTOA development is due to the actual ACL transection, and not the occurrence of a surgery. This data is currently being collected and will be analyzed soon.

The future direction of our research is to test the hypothesis that enhancing inflammation resolution will improve PTOA outcomes after ACL transection. We plan to inject exogenous, MaR1 and/or RvD1 to the surgical joint of male mice directly after their ACLT surgery. We will analyze the data collected from these mice compared to the ACLT mice that did not receive treatment and mice that receive a vehicle control

treatment. We predict that exogenous SPMs will help alleviate PTOA symptoms and progression. If results are significant, SPM administration could be considered as a potential therapeutic for PTOA prevention.

### **Conclusion**

In conclusion, the results of this study suggest that ACL injury does lead to joint inflammation and the release of SPMs. Our data suggests that SPMs are protective against PTOA development as loss of the receptor for MaR1, LGR6, worsened PTOA outcomes. However, it appears that endogenous SPMs are insufficient to prevent the development of PTOA. Despite this, it appears that SPMs still play a role in the process, and future studies should further investigate their potential therapeutic effects.

Additionally, sexual dimorphism was observed after ACLT, with female mice having less severe PTOA than male mice. This observation may be related to differences in SPMs production, providing support for the idea that SPMs may have a beneficial effect in preventing PTOA. Further studies are warranted to better understand the underlying mechanisms of SPMs in joint inflammation and the development of PTOA.

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**CURRICULUM VITAE**

