Am J Transl Res 2021;13(7):7667-7676
www.ajtr.org /ISSN:1943-8141/AJTR0127762

Original Article

Enrichment of inflammatory mediators in the synovial fluid is associated with slower progression of mild to moderate osteoarthritis in the porcine knee

Naga Padmini Karamchedu[1], Braden C Fleming[1], Jonah I Donnenfield[2], Benedikt L Proffen[2], Meggin Q Costa[1], Janine Molino[1], Martha M Murray[2]

[1]Department of Orthopaedics, Warren Alpert Medical School of Brown University/Rhode Island Hospital, Providence, RI, USA; [2]Division of Sports Medicine, Department of Orthopaedic Surgery, Boston Children's Hospital, Harvard Medical School, Boston, MA, USA

Received December 7, 2020; Accepted May 10, 2021; Epub July 15, 2021; Published July 30, 2021

Abstract: The roles that cytokines and matrix metalloproteinases play in the onset and progression of posttraumatic osteoarthritis (PTOA) remain a topic of debate. The study objective was to evaluate the concentrations of these inflammatory mediators during the development of mild to moderate PTOA in the porcine anterior cruciate ligament (ACL) surgical model. We hypothesized that there would be more animals with detectable mediators in the pigs that develop moderate PTOA (those receiving ACL reconstruction or untreated ACL transection) compared to those that develop mild PTOA (those receiving scaffold-enhanced ACL repair). 36 Yucatan minipigs underwent ACL transection and were randomized to: 1) no further treatment, 2) ACL reconstruction, or 3) scaffold-enhanced ACL repair. Synovial fluid samples were obtained pre-operatively, and at 1, 4, 12, 26 and 52 weeks post-operatively. The concentrations of inflammatory mediator in the synovial fluid samples were evaluated via multiplex assay. Macroscopic cartilage assessments were performed following euthanasia at 52 weeks. As found in prior studies, the repair group had significantly less cartilage damage than either the ACL transected or ACL reconstruction groups ($P<.03$). The presence and concentrations of the biomarkers were influenced by surgical group and time. In general, the concentrations of inflammatory mediators were higher in the repair group, which exhibited less cartilage damage than the other two treatment groups. While this finding disproved the hypotheses, these data suggest that the metabolic activity of the joints exhibiting less cartilage damage remained higher over the 52-week period than those that did not.

Keywords: Anterior cruciate ligament, posttraumatic osteoarthritis, cytokines, MMPs, biomarker, animal model

Introduction

Posttraumatic osteoarthritis (PTOA) rates have been reported to be as high as 80% a decade after anterior cruciate ligament (ACL) injury [1]. This is true whether the injured knee is biomechanically stabilized with a graft or not [2]. The biologic response to injury is thought to play a large role in the development of PTOA. The inflammatory cascades, including several members of the interleukin family, have been widely implicated to contribute to its development [3]. Pro-inflammatory cytokines have been reported in synovial fluid samples of ACL injured patients at three weeks (IL-1β, IL-6, IL-8 and TNF-α) [4] and 2 months after injury (IL-1α, IL-2, IL-6, IL-8, TNF-α) [5], as well as in patients with established osteoarthritis [6].

One mechanism by which the increased interleukins are thought to stimulate osteoarthritis is through the induction of matrix metalloproteinases [7, 8]. Matrix metalloproteinases (MMPs) are known to have activity against members of the collagen family, with MMP-1, MMP-3 and MMP-13 known to have specific activity against type II collagen, the primary collagen type found in articular cartilage. MMP-1, MMP-3 and MMP-13 are all thought to cause fibrillation and the associated loss of collagen that is the hallmark of osteoarthritis [9-11]. Thus, one target of current and future treatments for osteoarthritis is to quiet interleukin and MMP production, and hence the subsequent cartilage destruction after joint injury [12]. Anti-inflammatory drugs, including corticosteroid injection and non-steroidal anti-inflam-

Table 1. The 11 cytokines and 8 matrix metalloproteinases that were evaluated

Cytokines	MMPs
GMCSF	MMP-1
IL-1α	MMP-2
IL1-Ra	MMP-3
IL-2	MMP-7
IL-4	MMP-9
IL-6	MMP-10
IL-8	MMP-12
IL-10	MMP-13
IL-12	
IL-18	
TNF-α	

matory medications are mainstays in the treatment of mild to moderate symptomatic PTOA.

To evaluate causes of PTOA, large animal models offer a joint that is closer in scale to the human knee. They can be readily studied via synovial fluid aspirations at discrete time points after a surgically induced injury, and then compared to the damage seen after joint harvest. Recently, the Yucatan mini-pig model has been shown to reliably develop macroscopic cartilage changes after ACL transection [13-15]. In each of these prior studies, most animals treated with ACL transection alone, or ACL transection followed by ACL reconstruction, developed PTOA, particularly in the medial compartment. However, animals treated with a scaffold-enhanced ACL repair developed significantly less PTOA [13, 15].

The objective of the present study was to evaluate the interleukin and MMP concentrations (**Table 1**) in synovial fluid of knees over time during the development of mild to moderate PTOA after ACL injury. Based on current thinking, we hypothesized that: 1) there would be more animals with detectable levels of interleukins associated with the inflammatory cascade in the groups going on to develop moderate PTOA (ACL transection and ACL reconstruction) than the group going on to mild PTOA (scaffold-enhanced ACL repair), and 2) that the quantitative levels of the interleukins in the synovial fluid of the animals with detectable levels would also be higher in the two groups developing moderate PTOA. Given the relationship between interleukins and MMPs, we further hypothesized that: 3) there would be more animals with detectable levels of MMPs in the groups going on to develop moderate PTOA, and 4) the concentrations of MMPs in the synovial fluid of the animals with detectable levels would be highest in the groups developing moderate PTOA.

Materials and methods

Study design

The study was designed following the ARRIVE guidelines. Institutional animal care and use committee approvals were acquired prior to beginning the study (Protocol number: 15110-00175). Thirty six Yucatan mini-pigs in late adolescence [age (mean ± standard deviations): 15.3±1.6 months; weight: 52.1±4.6 kg] underwent ACL transection under anesthesia and were randomized to one of three experimental groups: 1) no treatment (ACLT group), 2) ACL reconstruction with bone-patellar tendon-bone allograft (ACLR group), and 3) scaffold-enhanced ACL repair using a scaffold combined with autologous blood (BE-R group) as previously described [15]. Synovial fluid aspirations were performed pre-operatively, and at 1, 4, 12, 26 and 52 weeks after surgery while the animal was under anesthesia. Macroscopic cartilage assessments were performed following euthanasia at 52 weeks. The macroscopic data from these animals have been previously reported in a paper evaluating gait changes post ACL surgery [15]. Details regarding animal husbandry, anesthesia, the surgical procedures, pain management and euthanasia have been previously reported [15]. No animals were excluded from any of the assigned treatments or analyses. All post-operative assessments were performed with the investigators blinded to the animal group assignment.

Synovial fluid aspiration

Prior to performing the ACL transection, the knee joint was irrigated with a 10 cc intra-articular injection of sterile normal saline while under anesthesia. Approximately 1 ml of the diluted synovial fluid was then aspirated with a 23-gauge needle. For postoperative synovial fluid collection with the animal under anesthesia, a 23-gauge needle was inserted into the joint through the patellar tendon. Fluid collected from the femoral notch region was centri-

fuged at 1300 relative centrifugal force (RCF), and the supernatant was stored at -80°C in 50 ul aliquots until analysis. If the initial aspiration was unsuccessful, the collection was repeated after a 10 cc phosphate buffered saline injection. At the time of aspiration, blood was also collected from the cranial vena cava and placed into 5 ml vacutainer serum separation tubes for 30 minutes. The serum was then centrifuged for 15 minutes at 3000 RCF to consolidate the cellular precipitate, and the supernatant was stored in 1.5 ml aliquots. The serum samples were used to establish the synovial fluid dilution as described below [16].

Multiplex assay

A custom multiplex assay kit (SPR#1178, Millipore, Burlington, MA) was used to determine the concentrations of 19 target proteins (11 cytokines and 8 MMPs) in the synovial fluid samples (**Table 1**). All synovial fluid samples were assayed in duplicate, and the average of these values was used. Fluorescent intensity data were acquired using a multiplex assay system (Bioplex-200; BioRAD, Hercules, CA). Concentrations were estimated by five parameters logistic regression of mean fluorescent intensity values using commercial software (Bioplex Manager; BioRAD, Hercules CA). Synovial fluid and serum concentrations of urea were normalized to correct for the dilution [16] by the lavage using the blood urea nitrogen (BUN) assay (ab83362, Cambridge, MA). All concentrations are expressed in picograms per milliliter (pg/ml).

Macroscopic articular cartilage analysis

Macroscopic damage of the articular cartilage surfaces was assessed immediately after euthanasia and joint harvest according to OARSI guidelines for sheep and goat [17] as a scoring system for the pig was not available. It should be noted that we have previously shown that this system is sensitive to differences in treatment groups when applied to the pig [13, 14]. Damages to six articular surfaces, including the medial femoral condyle, medial tibial plateau, lateral femoral condyle, lateral tibial plateau, femoral trochlea, and patella, were scored from 0 (i.e., normal) to 4 (i.e., large erosions down to subchondral bone) and reported separately. Scores from the four tibiofemoral surfaces were then summed to make up the total macroscopic score of the tibiofemoral joint, which could range from 0 to 16.

Statistical analyses

Data were imported into SAS version 9.4 (SAS Institute Inc., Cary, NC) for statistical analysis. Generalized estimating equations (GEE) were used to model (1) the presence of synovial fluid proteins, and (2) the synovial fluid protein concentrations as a function of animal within time and experimental group. Generalized linear regression models were used to model 52-week macroscopic damage as a function of experimental group. A logarithmic distribution was assumed for all cytokine and MMP concentration data and a logistic distribution was assumed for the presence/absence of synovial fluid proteins and 52-week macroscopic damage data. Classical sandwich estimation was used to adjust for any possible model misspecification. Pairwise comparisons between treatment groups were conducted via orthogonal contrasts where appropriate. The Holm-test was used to adjust for multiple comparisons to maintain a two-tailed family-wise alpha at 0.05. All modeling was performed using *PROC GLIMMIX* and all orthogonal contrasts were completed using the *lmeans* and *lsmestimate* statements. An adjusted P-value ≤ 0.05 was used to determine statistical significance. The primary analyses for comparison of the concentration of the MMPs and cytokines were conducted only for the MMPs and cytokines where at least two surgical groups had more than 60% of the samples with values above the detectable limit. These primary analyses only included the values above the detectable limit. Sensitivity analyses, where concentrations below the detectable limit of the assay were assigned a value equal to zero, were conducted to assess the robustness of the study findings.

Results

Cytokine presence in the synovial fluid after surgery (hypothesis 1)

There was a significant effect of surgical group on the proportion of animals with detectable levels of multiple cytokines, with post-hoc analyses finding differences between surgical groups in the number of knees that had detectable levels of GM-CSF, IL-1α, IL-1Ra, IL-2, IL-4, IL-6 and IL-10 for at least one post-operative

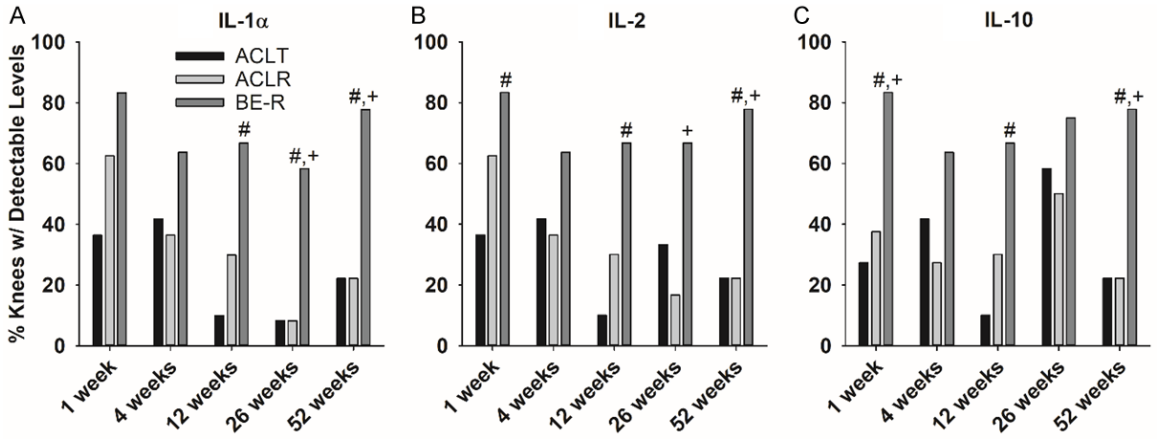

Figure 1. Percentage of knees in each surgical group that had detectable levels of interleukins. ACLT = ACL Transection, ACLR = ACL reconstruction, and BE-R = scaffold-enhanced ACL Repair. #denotes time points in which the BE-R group had significantly higher percentages of knees with detectable levels of cytokines compared to the ACLT group. + denotes time points where the BE-R group had significantly greater percentages than the ACLR group. Table S1 has the percentages with detectable levels for all cytokines evaluated.

Table 2. Concentrations (mean ± SD, pg/ml) and number of surgical joints (N) in which the cytokines (pg/ml) were above the detectable limit (excluding undetectable values)

Cytokines	ACLT	BE-R	ACLR	*P* value
IL-1Ra				0.01
1 week	317.76±515.09 (11)	925.15±792.50^{+} (12)	179.65±256.58 (8)	
IL-4				0.01
26 weeks	37.11±77.62 (10)	687.65±1372.08$^{\#}$ (12)	358.37±1197.02 (10)	
IL-6				0.01
1 week	379.79±170.57 (11)	684.0±528.43$^{\#}$ (12)	643.79±283.15$^{\#}$ (8)	
IL-12				<0.01
1 week	57.78±21.00 (11)	930.74±701.01^{+} (12)	55.58±34.36 (8)	
4 weeks	89.00±14.22 (12)	817.81±904.37$^{\#,+}$ (11)	112.37±107.12 (11)	
26 weeks	30.91±31.11 (10)	118.03±174.86 (12)	67.63±161.97 (10)	
IL-18				<0.01
1 week	719.93±934.72 (11)	2855.3±2683.79$^{\#,+}$ (12)	570.16±409.7 (8)	
4 weeks	2438.69±4537.69 (12)	4798.52±5339.20 (11)	2227.10±3008.99 (11)	
12 weeks	760.75±466.78 (10)	1303.77±1091.81 (12)	927.51±507.09 (10)	
26 weeks	533.58±454.07 (10)	923.80±695.36 (12)	873.65±823.77 (10)	
52 weeks	1275.3±875.41 (9)	1447.07±763.93 (8)	997±582 (9)	

ACLT = ACL Transection, ACLR = ACL reconstruction, and BE-R = scaffold-enhanced ACL Repair. *P* values represent the interaction term (treatment x time). If no interaction was found, then they represent the treatment effect. $^{\#}$P<0.05 for comparison to the ACLT group, $^{+}$P<0.05 for comparison to ACLR group. *P*-values for the interaction of surgical group and time-point are listed for comparisons where relevant. *P*-values for treatment effect are included when only one time point was considered.

time point (P<0.05 for all comparisons; **Figures 1**, S1; Table S1). In all cases, the BE-R group had a higher proportion of animals with detectable levels of cytokines which drove the observed statistical differences. There was no significant effect of surgical group on the numbers of animals with detectable levels of IL-8, IL-12, IL-18 or TNF-α in the joint fluid at any time point (Table S1).

Comparison of cytokine levels between surgical groups (hypothesis 2)

Cytokine concentrations were compared only when two or more surgical groups had sufficient (>60%) detectable samples at a time point and using only the samples with detectable levels (**Table 2**). One cytokine, IL-18, was present in at least 60% of the animals in all sur-

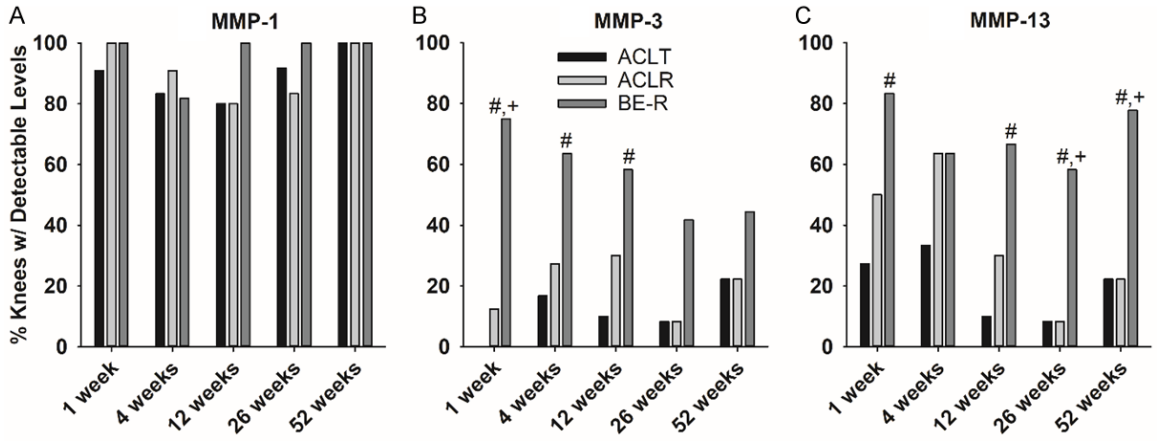

Figure 2. Percentage of knees in each surgical group that had detectable levels of MMP-1, MMP-3 and MMP-13. ACLT = ACL transection, ACLR = ACL reconstruction, and BE-R = scaffold-enhanced ACL repair. #denotes time points where the BE-R group had significantly higher percentages of knees with detectable levels of cytokines than the ACLT group. +denotes time points where BE-R group had significantly greater percentages than the ACLR group.

gical groups at all time points (Table S2). Other cytokines, including IL-1Ra, IL-4, IL-6, and IL-12, met the threshold of being present in at least 60% of the knees for at least one time point. The BE-R group had significantly higher concentrations of IL-1Ra, IL-6, IL-12 and IL-18 at one week (**Tables 2**, S2; Figure S2). At four weeks, IL-12 concentrations were significantly higher in the BE-R group, and at 26 weeks, IL-4 levels were higher in the BE-R group (**Tables 2**, S2; Figure S2).

MMP presence in the synovial fluid after surgery (hypothesis 3)

There was a significant difference between surgical groups in the proportion of animals that had detectable levels of multiple MMPs (MMP-2, MMP-3, MMP-7, MMP-10, MMP-12, and MMP-13) for at least one post-operative time point ($P<0.05$ for all comparisons; **Figures 2**, S3; Table S3). In all cases, the BE-R group had a higher proportion of animals with detectable levels of MMPs than either the ACLT group, ACLR group or both the ACLT and ACLR groups. There were no significant effects of surgical group on the proportion of animals with detectable levels of MMP-1 or MMP-9 in the joint fluid at any time point (**Figures 2**, S3; Table S3).

Comparison of MMP levels between surgical groups (hypothesis 4)

The only MMP that was present at detectable levels in at least 60% of the samples in all three surgical groups was MMP-1, which reached this threshold at all time points (**Figure 2**; Table S4). MMP-1 levels in those animals with detectable levels were significantly lower at one week in the BE-R group than in the ACLT or ACLR groups ($P<0.001$) (**Table 3**). There were no other significant differences between groups at the other time points (Figure S4; Table S4).

Macroscopic articular cartilage score

As reported previously [15], the BE-R group had less than half the amount of cartilage damage in the medial tibial plateau at 52 weeks than either the ACLT or ACLR group ($P=0.03$ for both comparisons; **Figure 3**; **Table 4**). There was also evidence that the BE-R group had less total articular cartilage damage at 52 weeks than either the ACLT or ACLR group ($P=0.06$ for both comparisons; **Figure 3**; **Table 4**) [15]. The medial tibial plateau had cartilage erosions down to the subchondral bone in three of the ACL transected knees, four of the ACL reconstructed knees and zero of the BE-R knees. There were no significant differences in the contralateral macroscopic damage scores between the groups.

Discussion

In this study, we determined that the concentrations of various cytokines and MMPs changed over time, indicating that the metabolic activity of the joint was affected post-surgery. Furthermore, the concentrations of the

Table 3. Concentrations (mean ± SD, pg/ml) and number of surgical joints (N) in which the MMPs were above the detectable limit

Matrix Metalloproteinases (MMPs)				*P* value
MMP-1	ACLT	BE-R	ACLR	<0.001
1 week	253.30±83.57 (8)	199.2±82.85^{+} (12)	332.07±130.34 (8)	
4 weeks	237.73±72.93 (11)	295.40±148.45 (11)	336.42±57.76 (11)	
12 weeks	266.87±99.57 (12)	181.33±102.8 (12)	208.88±87.01 (10)	
26 weeks	106.69±54.49 (10)	110.61±38.69 (12)	131.02±35.48 (10)	
52 weeks	111.31±34.4 (9)	135.93±43.93 (8)	94.57±32.27 (9)	

ACLT = ACL Transection, ACLR = ACL reconstruction, and BE-R = scaffold-enhanced ACL Repair. $^{+}$P<0.05 for comparison to the ACLR group. *P*-values for the interaction of surgical group and time-point are listed.

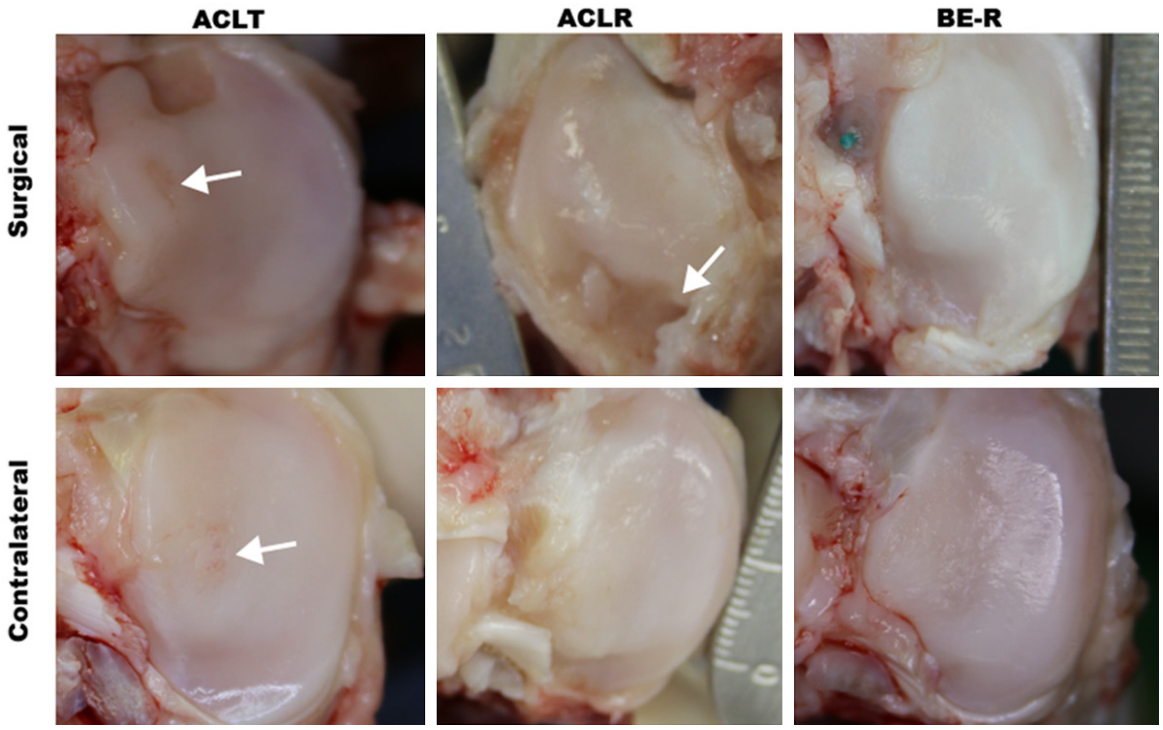

Figure 3. Macroscopic cartilage damage in the medial tibial plateau cartilage of surgical and contralateral knees. These images represent the mean macroscopic cartilage damage scores presented in **Table 4**. The top of the image is anterior, and the bottom is posterior. Arrows point to the cartilage lesions. The surgical limb of the ACLT group cartilage shows a small fissure while the contralateral limb shows some surface roughening. The surgical limb of ACLR specimen shows a large erosion down to the bone.

Table 4. Macroscopic damage scores (mean±SD) for each region for all groups at 52 weeks

	ACLT		ACLR		BE-R	
	Surgical	Contralateral	Surgical	Contralateral	Surgical	Contralateral
Medial Tibial Plateau	1.6±0.42	0.4±0.67	2.1±1.38	0.5±0.67	0.7±0.67$^{\#,+}$	0.7±0.65
Total Macroscopic Score	7.8±2.79	1.8±1.27	7.5±2.68	1.4±2.68	4.8±3.04$^{\#\#,++}$	1.9±1.24

ACLT = ACL Transection, ACLR = ACL reconstruction, and BE-R = scaffold-enhanced ACL Repair (adapted from Karamchedu et al [15]). $^{\#}$P-adj<0.05 compared to ACLT; $^{\#\#}$P-adj = 0.06 compared to ACLT; $^{+}$P-adj<0.05 compared to ACLR; $^{++}$P-adj = 0.06 compared to ACLR.

cytokines and MMPs were influenced by the surgical treatment group. Contrary to our hypotheses, the cytokine and MMP levels were generally greater in the group exhibiting less

macroscopic cartilage damage (BE-R), when compared to those presenting greater cartilage damage (ACLR and ACLT). The extent of the macroscopic cartilage damage for each group [15] was consistent with the results of our prior studies [13, 14].

Inflammation has been increasingly implicated in the development of osteoarthritis [1, 18-20]. Multiple investigators have reported increased levels of interleukins in the synovial fluid of patients with osteoarthritis [6, 21-23]. In vitro studies have demonstrated that the treatment of cells or tissue explants with various interleukins can result in production of matrix-metalloproteinases by the chondrocytes leading to matrix degradation [7, 24]. For several of the interleukin family members, young knock-out mice exhibit less disease [25-27]. All these studies support the premise that inflammation is responsible for osteoarthritis. However, in the present study, detectable concentrations of interleukins, both "pro-" and "anti-inflammatory" cytokines, were present in the animals developing less severe cartilage changes (BE-R group). Likewise, detectable levels of MMPs were also present in more knees of this group. These observations challenge the prior body of evidence supporting our original hypotheses. To reconcile our findings, we postulate that the increase in interleukin concentration in the synovial fluid is the joint's attempt to repair the damaged cartilage, and that this response may be triggered by both native collagen molecules released into the joint fluid as cartilage breakdown occurs and by the surgical placement of collagen molecules in the joint, as with scaffold-enhanced ACL repair (BE-R group). In the case of progressive osteoarthritis, the repair response may be present but not sufficient to reverse the damage. However, in knees where the repair response is prolonged, as seen in the BE-R knees, the prolonged response may minimize the rate of progression of the damage. The postulate that the increased presence of interleukins may be reparative can be reconciled with experiments in aged mice that demonstrated worse osteoarthritis when an individual interleukin molecule was knocked out [28]. In addition, the concept that a balanced, sustained upregulation of the interleukin response is important in controlling the rate of progression of osteoarthritis would also be consistent with recent results from a randomized control trial, where intra-articular corticosteroid use (which essentially dampens the interleukin response) led to faster progression of osteoarthritis [29]. Recently published results from the Osteoarthritis Initiative indicate that the use of intra-articular corticosteroids resulted in an acceleration of osteoarthritic changes in human patients [30]. Patients have also been shown to progress to total knee arthroplasty at a six-fold greater rate (30% vs 5%) after corticosteroid injections [31]. Finally, the concept that inflammation may be associated with the degree of osteoarthritis present, but not the rate of progression, is supported further by two systematic reviews [32, 33].

In the same animals [15], we found that the surgical treatment (BE-R) resulting in the lowest amount of macroscopic cartilage damage exhibited greater asymmetry in load-related gait parameters than the other surgical groups (ACLR and ACLT). This finding suggests that increased off-loading of the surgical knee may be associated with a slower rate of PTOA development. In the current analysis, we found that the concentrations of several of the cytokines and MMPs were higher in the group with less cartilage damage (BE-R). Therefore, the decreased weight bearing in gait may be due to inflammatory factors causing pain. Studies are currently underway to explore the interactions between gait, cytokine/MMP production, and cartilage health.

The current study has several strengths. First, we utilized the porcine model, which has anatomical and biomechanical similarities to the human knee [34-36]. While the tibiofemoral cartilage of the porcine knee may be thinner than that of the human knee [37], it is closer to the human knee when compared to other large animal models [38]. The porcine model also exhibits cartilage damage that mimics the pattern seen in humans but at an accelerated rate [13].

Another advantage of a large animal model to study PTOA is that it enabled us to perform a longitudinal assessment following a controlled surgical insult that initiates PTOA at a defined time point. It also allowed us to perform functional assessments [15], and to acquire synovial fluid samples at discrete time intervals following injury. At 52 weeks, the animals were euthanized so that direct assessments of the

articular cartilage damage could be made relative to the metabolic changes observed in the synovial fluid that are not conducive for human trials.

There are several limitations in this study. First, only a limited number of cytokines and MMPs were assayed. While a limited number of pro- and anti-inflammatory cytokines were assayed, we did not measure the presence and concentrations of tissue inhibitors of metalloproteinases (TIMPs) in conjunction with the eight MMPs that were studied. Therefore, the net effects of these cytokines and MMPs on long-term cartilage health remain unclear. Nonetheless, these data suggest that the group exhibiting less macroscopic damage of their surgical knees may be more metabolically active than the groups that did not.

Obtaining neat synovial fluid samples when aspirating the joints proved to be challenging in some of the animals, particularly at time points after the surgical swelling subsided. For each animal, we attempted to get a neat sample. However, when unsuccessful, lavage with phosphate buffered saline was used to obtain fluid. To account for the lavage, we normalized the dilutions by comparing the urea concentration in the serum with that of the synovial fluid sample [16].

As with most pre-clinical models, the pig is a quadruped animal and thus the limb loading conditions are different. Therefore, the results may not directly translate to the human condition. For this study, adolescent animals were used at the time of the surgically induced injury even though osteoarthritis generally afflicts older adults. As joint metabolism decreases with age, it is possible that the interleukins and MMP concentrations could be different in older adults. However, ACL injuries are most common in adolescents; thus, this model represents an important age group to study the joint response to this injury. Another limitation to the porcine model was that the ACL injury was surgically induced. A capsulotomy was required to access the ACL, which in turn could affect joint metabolism. Nonetheless, all three groups required capsulotomy, which minimizes the concern that this method could affect the group comparisons. Furthermore, the ACL transection procedure is an over-simplification of an ACL injury as no tibiofemoral compressive overload was required. However, ACL transection initiates PTOA in this model much like that seen in humans [13]. It also allowed us to control confounding factors that could also influence disease progression, including meniscal injuries or chondral lesions.

Finally, the sample size was a sample of convenience. The sample size for this study, 12 animals per group, was established *a priori* to detect differences in gait parameters [15]. It is possible that additional significant findings could have been found with more animals in each group. Nonetheless, several interesting significant differences were found with the given sample size.

In summary, we found that the presence and concentrations of the cytokines and MMPs under study were influenced by both surgical group and time. In general, the concentrations of pro- and anti-inflammatory mediators were generally higher in the group exhibiting less cartilage damage than the two treatment groups that exhibited more cartilage damage. These data suggest that enrichment of inflammatory mediators in the synovial fluid of joints is associated with slower progression of mild to moderate osteoarthritis.

Acknowledgements

The authors would like to thank Scott McAllister, Jakob Sieker MD, Jillian Beveridge PhD, Kaitlyn Chin, Kimberly Waller, Veronica Bouvier, Roxanne Burrill, Lara Helwig, DVM, and Tiffany Borgeson, DVM for their assistance with animal handling, surgical and tissue collection procedures. The investigation was supported by the National Institutes of Health (NIAMS R01-AR056834 & R01-AR065462; NIGMS P30-GM122732), and the Lucy Lippitt Endowment.

Disclosure of conflict of interest

It should be noted that Dr. Murray is a founder, paid consultant and equity holder, Dr. Proffen is a paid consultant and equity holder, and Dr. Fleming is a founder of Miach Orthopaedics, Inc, which was formed to upscale production of a scaffold for ACL repair and is related to one of the ACL procedures described herein. Drs. Murray and Proffen maintain a conflict-of-interest management plan approved by Boston Children's Hospital

and Harvard Medical School. Dr. Fleming also maintains a conflict-of-interest management plan with Rhode Island Hospital with similar oversight.

Address correspondence to: Dr. Braden C Fleming, Department of Orthopaedics, Warren Alpert Medical School of Brown University/Rhode Island Hospital, Coro West, Suite 402, 1 Hoppin St, Providence, RI 02903, USA. Tel: 401-316-4737; E-mail: braden_fleming@brown.edu

References

[1] Lie MM, Risberg MA, Storheim K, Engebretsen L and Oiestad BE. What's the rate of knee osteoarthritis 10 years after anterior cruciate ligament injury? An updated systematic review. Br J Sports Med 2019; 53: 1162-1167.

[2] Von Porat A, Roos EM and Roos H. High prevalence of osteoarthritis 14 years after an anterior cruciate ligament tear in male soccer players: a study of radiographic and patient relevant outcomes. Ann Rheum Dis 2004; 63: 269-273.

[3] Woodell-May JE and Sommerfeld SD. Role of inflammation and the immune system in the progression of osteoarthritis. J Orthop Res 2020; 38: 253-257.

[4] Sward P, Frobell R, Englund M, Roos H and Struglics A. Cartilage and bone markers and inflammatory cytokines are increased in synovial fluid in the acute phase of knee injury (hemarthrosis)--a cross-sectional analysis. Osteoarthritis Cartilage 2012; 20: 1302-1308.

[5] Amano K, Huebner JL, Stabler TV, Tanaka M, McCulloch CE, Lobach I, Lane NE, Kraus VB, Ma CB and Li X. Synovial fluid profile at the time of anterior cruciate ligament reconstruction and its association with cartilage matrix composition 3 years after surgery. Am J Sports Med 2018; 46: 890-899.

[6] Beekhuizen M, Gierman LM, van Spil WE, Van Osch GJ, Huizinga TW, Saris DB, Creemers LB and Zuurmond AM. An explorative study comparing levels of soluble mediators in control and osteoarthritic synovial fluid. Osteoarthritis Cartilage 2013; 21: 918-922.

[7] Hashimoto K, Otero M, Imagawa K, de Andres MC, Coico JM, Roach HI, Oreffo RO, Marcu KB and Goldring MB. Regulated transcription of human matrix metalloproteinase 13 (MMP13) and interleukin-1beta (IL1B) genes in chondrocytes depends on methylation of specific proximal promoter CpG sites. J Biol Chem 2013; 288: 10061-10072.

[8] Mitchell PG, Magna HA, Reeves LM, Lopresti-Morrow LL, Yocum SA, Rosner PJ, Geoghegan KF and Hambor JE. Cloning, expression, and type II collagenolytic activity of matrix metalloproteinase-13 from human osteoarthritic cartilage. J Clin Invest 1996; 97: 761-768.

[9] Wolfe GC, MacNaul KL, Buechel FF, McDonnell J, Hoerrner LA, Lark MW, Moore VL and Hutchinson NI. Differential in vivo expression of collagenase messenger RNA in synovium and cartilage. Quantitative comparison with stromelysin messenger RNA levels in human rheumatoid arthritis and osteoarthritis patients and in two animal models of acute inflammatory arthritis. Arthritis Rheum 1993; 36: 1540-1547.

[10] Neuhold LA, Killar L, Zhao W, Sung ML, Warner L, Kulik J, Turner J, Wu W, Billinghurst C, Meijers T, Poole AR, Babij P and DeGennaro LJ. Postnatal expression in hyaline cartilage of constitutively active human collagenase-3 (MMP-13) induces osteoarthritis in mice. J Clin Invest 2001; 107: 35-44.

[11] Reboul P, Pelletier JP, Tardif G, Cloutier JM and Martel-Pelletier J. The new collagenase, collagenase-3, is expressed and synthesized by human chondrocytes but not by synoviocytes. A role in osteoarthritis. J Clin Invest 1996; 97: 2011-2019.

[12] Lohmander LS, Hoerrner LA, Dahlberg L, Roos H, Bjornsson S and Lark MW. Stromelysin, tissue inhibitor of metalloproteinases and proteoglycan fragments in human knee joint fluid after injury. J Rheumatol 1993; 20: 1362-1368.

[13] Murray MM and Fleming BC. Use of a bioactive scaffold to stimulate anterior cruciate ligament healing also minimizes posttraumatic osteoarthritis after surgery. Am J Sports Med 2013; 41: 1762-1770.

[14] Fleming BC, Proffen BL, Vavken P, Shalvoy MR, Machan JT and Murray MM. Increased platelet concentration does not improve functional graft healing in bio-enhanced ACL reconstruction. Knee Surg Sports Traumatol Arthrosc 2015; 23: 1161-1170.

[15] Karamchedu NP, Murray MM, Sieker JT, Proffen BL, Portilla G, Costa MQ, Molino J and Fleming BC. Bridge-enhanced anterior cruciate ligament repair leads to greater limb asymmetry and less cartilage damage than untreated ACL transection or ACL reconstruction in the porcine model. Am J Sports Med 2021; 49: 667-674.

[16] Kraus VB, Huebner JL, Fink C, King JB, Brown S, Vail TP and Guilak F. Urea as a passive transport marker for arthritis biomarker studies. Arthritis Rheum 2002; 46: 420-427.

[17] Little CB, Smith MM, Cake MA, Read RA, Murphy MJ and Barry FP. The OARSI histopathology initiative - recommendations for histological assessments of osteoarthritis in sheep and goats. Osteoarthritis Cartilage 2010; 18 Suppl 3: S80-92.

[18] He Y, Li Z, Alexander PG, Ocasio-Nieves BD, Yocum L, Lin H and Tuan RS. Pathogenesis of osteoarthritis: risk factors, regulatory pathways in chondrocytes, and experimental models. Biology (Basel) 2020; 9: 194.
[19] Tourville TW, Poynter ME, DeSarno MJ, Struglics A and Beynnon BD. Relationship between synovial fluid ARGS-aggrecan fragments, cytokines, MMPs, and TIMPs following acute ACL injury: a cross-sectional study. J Orthop Res 2015; 33: 1796-1803.
[20] Lohmander LS, Atley L, Pietka TA and Eyre DR. The release of cross linked proteins from type II collagen in human synovial fluid is increased soon after injury. Arthritis Rheum 2003; 48: 3130-3139.
[21] Wiegertjes R, van de Loo FAJ and Blaney Davidson EN. A roadmap to target interleukin-6 in osteoarthritis. Rheumatol 2020; 59: 2681-2694.
[22] Wang ZW, Chen L, Hao XR, Qu ZA, Huang SB, Ma XJ, Wang JC and Wang WM. Elevated levels of interleukin-1beta, interleukin-6, tumor necrosis factor-alpha and vascular endothelial growth factor in patients with knee articular cartilage injury. World J Clin Cases 2019; 7: 1262-1269.
[23] Mabey T, Honsawek S, Tanavalee A, Yuktanandana P, Wilairatana V and Poovorawan Y. Plasma and synovial fluid inflammatory cytokine profiles in primary knee osteoarthritis. Biomarkers 2016; 21: 639-644.
[24] Liacini A, Sylvester J, Li WQ, Huang W, Dehnade F, Ahmad M and Zafarullah M. Induction of matrix metalloproteinase-13 gene expression by TNF-alpha is mediated by MAP kinases, AP-1, and NF-kappaB transcription factors in articular chondrocytes. Exp Cell Res 2003; 288: 208-217.
[25] Wu X, Cao L, Li F, Ma C, Liu G and Wang Q. Interleukin-6 from subchondral bone mesenchymal stem cells contributes to the pathological phenotypes of experimental osteoarthritis. Am J Transl Res 2018; 10: 1143-1154.
[26] Latourte A, Cherifi C, Maillet J, Ea HK, Bouaziz W, Funck-Brentano T, Cohen-Solal M, Hay E and Richette P. Systemic inhibition of IL-6/Stat3 signalling protects against experimental osteoarthritis. Ann Rheum Dis 2017; 76: 748-755.
[27] Cornelissen F, Mus AM, Asmawidjaja PS, van Hamburg JP, Tocker J and Lubberts E. Interleukin-23 is critical for full-blown expression of a non-autoimmune destructive arthritis and regulates interleukin-17A and RORgammat in gammadelta T cells. Arthritis Res Ther 2009; 11: R194.
[28] de Hooge AS, van de Loo FA, Bennink MB, Arntz OJ, de Hooge P and van den Berg WB. Male IL-6 gene knock out mice developed more advanced osteoarthritis upon aging. Osteoarthritis Cartilage 2005; 13: 66-73.
[29] McAlindon TE, LaValley MP, Harvey WF, Price LL, Driban JB, Zhang M and Ward RJ. Effect of intra-articular triamcinolone vs saline on knee cartilage volume and pain in patients with knee osteoarthritis: a randomized clinical trial. JAMA 2017; 317: 1967-1975.
[30] Zeng C, Lane NE, Hunter DJ, Wei J, Choi HK, McAlindon TE, Li H, Lu N, Lei G and Zhang Y. Intra-articular corticosteroids and the risk of knee osteoarthritis progression: results from the osteoarthritis initiative. Osteoarthritis Cartilage 2019; 27: 855-862.
[31] Wijn SRW, Rovers MM, van Tienen TG and Hannink G. Intra-articular corticosteroid injections increase the risk of requiring knee arthroplasty. Bone Joint J 2020; 102-B: 586-592.
[32] Jin X, Beguerie JR, Zhang W, Blizzard L, Otahal P, Jones G and Ding C. Circulating C reactive protein in osteoarthritis: a systematic review and meta-analysis. Ann Rheum Dis 2015; 74: 703-710.
[33] Kerkhof HJ, Bierma-Zeinstra SM, Castano-Betancourt MC, de Maat MP, Hofman A, Pols HA, Rivadeneira F, Witteman JC, Uitterlinden AG and van Meurs JB. Serum C reactive protein levels and genetic variation in the CRP gene are not associated with the prevalence, incidence or progression of osteoarthritis independent of body mass index. Ann Rheum Dis 2010; 69: 1976-1982.
[34] Boguszewski DV, Shearn JT, Wagner CT and Butler DL. Investigating the effects of anterior tibial translation on anterior knee force in the porcine model: is the porcine knee ACL dependent? J Orthop Res 2011; 29: 641-646.
[35] Proffen BL, McElfresh M, Fleming BC and Murray MM. A comparative anatomical study of the human knee and six animal species. Knee 2012; 19: 469-476.
[36] Xerogeanes JW, Fox RJ, Takeda Y, Kim HS, Ishibashi Y, Carlin GJ and Woo SL. A functional comparison of animal anterior cruciate ligament models to the human anterior cruciate ligament. Ann Biomed Engin 1998; 26: 345-352.
[37] Kiapour AM, Shalvoy MR, Murray MM and Fleming BC. Validation of porcine knee as a sex-specific model to study human anterior cruciate ligament disorders. Clin Orthop Relat Res 2015; 473: 639-699.
[38] Athanasiou KA, Rosenwasser MP, Buckwalter JA, Malinin TI and Mow VC. Interspecies comparisons of in situ intrinsic mechanical properties of distal femoral cartilage. J Orthop Res 1991; 9: 330-340.

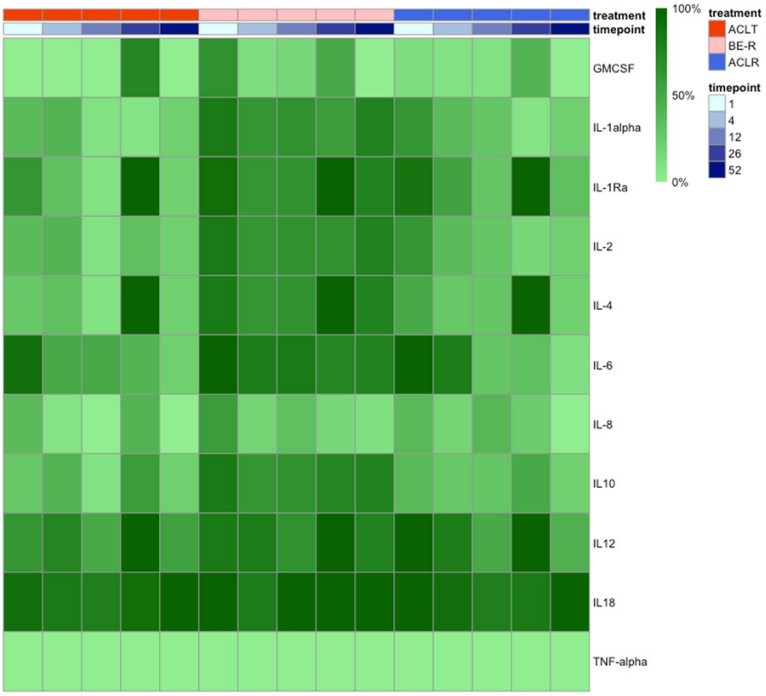

Figure S1. The percentages of synovial fluid samples with detectable levels of cytokines are shown in the heat map for each surgical group at 1, 4, 12, 26, and 52 weeks. Note that 0% of TNF-alpha samples were above the detectable threshold. Statistically significant comparisons are best interpreted with the data in Table S1.

Table S1. Differences in the proportion of animals with detectable levels of tested cytokines. Numerator is the number of animals with a detectable level of cytokine at that time point and the denominator is the total number of animals where fluid was obtained

Cytokine	Time	ACLT	BE-R	ACLR	*P*-Value
GMCSF	Week 1	0/11 (0.0)	8/12 (66.7)$^{\#,+}$	1/8 (12.5)	<0.001
	Week 4	0/12 (0.0)	2/11 (12.2)	1/11(9.1)	0.29
	Week 12	0/10 (0.0)	2/12 (16.7)	1/10 (10.0)	0.76
	Week 26	9/12 (75.0)	6/12 (50.0)	5/12 (41.7)	0.33
	Week 52	0/9 (0.0)	0/9 (0.0)	0/9 (0.0)	N/A
IL-1α	Week 1	4/11 (36.4)	10/12 (83.3)	5/8 (62.5)	0.07
	Week 4	5/12 (41.7)	7/11 (63.7)	4/11 (36.4)	0.47
	Week 12	1/10 (10.0)	8/12 (66.7)$^{\#}$	3/10 (30.0)	0.02
	Week 26	1/12 (8.3)	2/12 (58.3)$^{\#,+}$	1/12 (8.3)	0.007
	Week 52	2/9 (22.2)	7/9 (77.8)$^{\#,+}$	2/9 (22.2)	0.03
IL-1Ra	Week 1	7/11 (63.6)	11/12 (91.7)	7/8 (87.5)	0.23
	Week 4	4/12 (33.3)	7/11 (63.6)	6/11 (54.6)	0.39
	Week 12	1/10 (10.0)	8/12 (66.7)$^{\#}$	3/10 (30.0)	0.02
	Week 26	12/12 (100)	12/12 (100)	12/12 (100)	N/A
	Week 52	2/9 (22.2)	7/9 (77.8)$^{\#}$	3/9 (33.3)	0.09
IL-2	Week 1	4/11 (36.4)	10/12 (83.3)$^{\#}$	5/8 (62.5)	0.07
	Week 4	5/12 (41.7)	7/11 (63.6)	4/11 (36.4)	0.47
	Week 12	1/10 (10.0)	8/12 (66.7)$^{\#}$	3/10 (30.0)	0.02
	Week 26	4/12 (33.3)	8/12 (66.7)$^{+}$	2/12 (16.7)	0.06
	Week 52	2/9 (22.2)	7/9 (77.8)$^{\#,+}$	2/9 (22.2)	0.03
IL-4	Week 1	3/11 (27.3)	10/12 (83.3)$^{\#}$	4/8 (50.0)	0.03

	Week 4	4/12 (33.3)	7/11 (63.6)	3/11 (27.3)	0.25
	Week 12	1/10 (10.0)	8/12 (66.7)[#]	3/10 (30.0)	0.02
	Week 26	12/12 (100)	12/12 (100)	12/12 (100)	N/A
	Week 52	2/9 (22.2)	7/9 (77.8)[#,+]	2/9 (22.2)	0.03
IL-6	Week 1	10/11 (90.9)	12/12 (100)	8/8 (100)	0.61
	Week 4	6/12 (50.0)	9/11 (81.8)	9/11 (81.8)	0.21
	Week 12	5/10 (50.0)	10/12 (83.3)[+]	3/10 (30.0)	0.05
	Week 26	5/12 (41.7)	9/12 (75.0)[+]	4/12 (33.3)	0.17
	Week 52	2/9 (22.2)	7/9 (77.8)[#,+]	1/9 (11.1)	0.02
IL-8	Week 1	4/11 (36.4)	7/12 (58.3)	3/8 (37.5)	0.60
	Week 4	1/12 (8.3)	2/11 (18.2)	2/11 (18.2)	0.71
	Week 12	0/10 (0.0)	4/12 (33.3)	4/10 (40.0)	0.09
	Week 26	5/12 (41.7)	2/12 (16.7)	3/12 (25.0)	0.53
	Week 52	0/9 (0.0)	1/9 (11.1)	0/9 (0.0)	0.99
IL-10	Week 1	3/11 (27.3)	10/12 (83.3)[#,+]	3/8 (37.5)	0.02
	Week 4	5/12 (41.7)	7/11 (63.6)	3/11 (27.3)	0.24
	Week 12	1/10 (10.0)	8/12 (66.7)[#]	3/10 (30.0)	0.02
	Week 26	7/12 (58.3)	9/12 (75.0)	6/12 (50.0)	0.58
	Week 52	2/9 (22.2)	7/9 (77.8)[#,+]	2/9 (22.2)	0.03
IL-12	Week 1	7/11 (63.6)	10/12 (83.3)	8/8 (100)	0.15
	Week 4	9/12 (75.0)	9/11 (81.8)	9/11 (81.8)	0.99
	Week 12	5/10 (50.0)	8/12 (66.7)	5/10 (50.0)	0.74
	Week 26	12/12 (100)	12/12 (100)	12/12 (100)	N/A
	Week 52	5/9 (55.6)	7/9 (77.8)	4/9 (44.4)	0.49
IL-18	Week 1	10/11 (90.9)	12/12 (100)	8/8 (100)	0.61
	Week 4	10/12 (83.3)	9/11 (81.8)	10/11 (90.9)	0.99
	Week 12	8/10 (80.0)	12/12 (100)	8/10 (80.0)	0.23
	Week 26	11/12 (91.7)	12/12 (100)	10/12 (83.3)	0.76
	Week 52	9/9 (100)	9/9 (100)	9/9 (100)	N/A

P values in table are for the overall effect of group based on the GEE analysis at each time point; [#]post-hoc analysis with Holm test *p*-value adjustment for multiple comparisons demonstrated significant difference in comparison to the ACLT group with P<0.05; [+]post-hoc analysis with Holm test *p*-value adjustment demonstrated significant difference in comparison to the ACLR group.

Table S2. Concentrations of cytokines (mean ± SD, pg/ml) with the minimum detectable concentration set to zero and number of surgical joints (N) involved

Cytokines	Time	ACLT	BE-R	ACLR
GMCSF	Preop	0±0 (8)	0±0 (9)	0±0 (10)
	1 week	0±0 (11)	49.3±58.65 (12)	4.82±13.62 (8)
	4 weeks	0±0 (12)	4.66±10.37 (11)	2.82±9.35 (11)
	12 weeks	0±0 (10)	5.23±12.42 (12)	2.87±9.07 (10)
	26 weeks	1133.61±3857.55 (12)	7.31±9.01 (12)	12.64±22.55 (12)
	52 weeks	0±0 (9)	0±0 (9)	0±0 (9)
IL-1α	Preop	3.63±10.22 (8)	1.71±5.1 (9)	0.45±1.4 (10)
	1 week	4.77±7.63 (11)	211.23±193.63 (12)	14.35±13.52 (8)
	4 weeks	17.07±35.42 (12)	222.39±286.41 (11)	22.38±43.42 (11)
	12 weeks	3.48±10.97 (10)	53.01±71.59 (12)	17.51±33.25 (10)
	26 weeks	2.08±7.18 (12)	32.1±52.8 (12)	13.74±47.56 (12)
	52 weeks	6.04±12.05 (9)	42.98±50.53 (9)	9.84±23.79 (9)

IL-1Ra	Preop	7.8±22.03 (8)	4.96±14.86 (9)	0.01±0 (10)
	1 week	202.21±429.99 (11)	848.05±801.43 (12)	157.2±245.89 (8)
	4 weeks	82.55±173.34 (12)	1262.31±2041.7 (11)	72.88±117.18 (11)
	2 weeks	7.88±24.88 (10)	163.78±270.81 (12)	41.28±77.5 (10)
	26 weeks	17.06±18.51 (12)	104.84±181.09 (12)	55.5±151.98 (12)
	52 weeks	17.54±36.83 (9)	126.53±147.21 (9)	26.56±57.4 (9)
IL-2	Preop	18.3±51.74 (8)	6.29±18.83 (9)	3.77±11.88 (10)
	1 week	30.6±47.87 (11)	1219.75±1126.92 (12)	62.34±74.47 (8)
	4 weeks	101.94±233.89 (12)	1763.4±2492.06 (11)	130.43±270.67 (11)
	12 weeks	22.55±71.26 (10)	337.03±532.38 (12)	98.35±189.17 (10)
	26 weeks	16.96±39.85 (12)	201.72±346.3 (12)	87.14±300.59 (12)
	52 weeks	55.03±113 (9)	234.74±273.68 (9)	59.99±143.25 (9)
IL-4	Preop	34.89±98.66 (8)	23.8±51.29 (9)	0.01±0 (10)
	1 week	40.33±74.01 (11)	8037.34±8751.47 (12)	97.61±140.68 (8)
	4 weeks	285.26±730.39 (12)	8259.98±12360.02 (11)	421.41±968.04 (11)
	12 weeks	52.75±166.78 (10)	1228.15±2041.6 (12)	285.43±562.43 (10)
	26 weeks	37.11±77.62 (12)	687.65±1372.08 (12)	358.37±1197.02 (12)
	52 weeks	100.36±205.09 (9)	809.25±1228.41 (9)	153.96±387.1 (9)
IL-6	Preop	9.06±25.6 (8)	0.01±0 (9)	0.01±0 (10)
	1 week	337.08±196.68 (11)	684±528.43 (12)	643.79±283.15 (8)
	4 weeks	102.49±164.86 (12)	784.08±970.04 (11)	369.39±332.88 (11)
	12 weeks	70.09±103.57 (10)	155.89±179.98 (12)	34.98±64.75 (10)
	26 weeks	5.28±10.5 (12)	63.88±121.64 (12)	30.61±101.16 (12)
	52 weeks	10.49±21.44 (9)	129.97±132.11 (9)	13.52±40.52 (9)
IL-8	Preop	7.6±21.49 (8)	0±0 (9)	5.57±17.6 (10)
	1week	50.32±95.73 (11)	280.89±669.83 (12)	377.12±754.37 (8)
	4 weeks	0±0 (12)	20.3±47.72 (11)	13.74±31.1 (11)
	12 weeks	0±0 (10)	10.92±17 (12)	6.11±7.98 (10)
	26 weeks	11.08±16.56 (12)	1.59±3.79 (12)	4.4±12.47 (12)
	52 weeks	0±0 (9)	6.49±19.48 (9)	0±0 (9)
IL-10	Preop	15.71±44.4 (8)	3.09±9.23 (9)	0.01±0 (10)
	1 week	17.71±31.58 (11)	1093.08±1069.87 (12)	22.18±39.41 (8)
	4 weeks	64.85±143.53 (12)	1081.57±1519.2 (11)	80.94±174.01 (11)
	12 weeks	16.66±52.65 (10)	221.92±360.98 (12)	69±130.54 (10)
	26 weeks	10.82±20.14 (12)	133.18±254.27 (12)	63.44±215.37 (12)
	52 weeks	38.4±76.25 (9)	145.98±194.93 (9)	36.72±88.31 (9)
IL-12	Preop	11.38±32.15 (8)	10.35±21.22 (9)	3.33±10.5 (10)
	1 week	36.77±33.38 (11)	775.62±730.29 (12)	55.58±34.36 (8)
	4 weeks	66.75±97.57 (12)	669.12±873.93 (11)	91.94±106.05 (11)
	12 weeks	24.39±33.81 (10)	119.06±170.68 (12)	48.07±68.64 (10)
	26 weeks	30.91±31.11 (12)	118.03±174.86 (12)	67.63±161.97 (12)
	52 weeks	37.48±46.56 (9)	138.57±151.43 (9)	39.45±71.73 (9)
IL-18	Preop	670.8±1066.04 (8)	560.87±629.69 (9)	200.91±121.57 (10)
	1 week	654.48±912.93 (11)	2855.3±2683.79 (12)	570.16±409.7 (8)
	4 weeks	2069.75±4216.81 (12)	3926.07±5154.95 (11)	2024.63±2932.49 (11)
	12 weeks	608.6±521.87 (10)	1303.77±1091.81 (12)	742.01±594.08 (10)
	26 weeks	489.12±459.52 (12)	923.81±695.36 (12)	728.05±819.06 (12)
	52 weeks	1275.3±875.41 (9)	1447.07±763.93 (9)	997±582 (9)
TNF-α	Preop	0±0 (8)	0±0 (9)	0±0 (10)

1 week	0±0 (11)	18.18±39.04 (12)	0±0 (8)
4 weeks	0±0 (12)	0±0 (11)	0±0 (11)
12 weeks	3.04±9.62 (10)	0±0 (12)	0±0 (10)
26 weeks	0±0 (12)	0±0 (12)	0±0 (12)
52 weeks	0±0 (9)	0±0 (9)	0±0 (9)

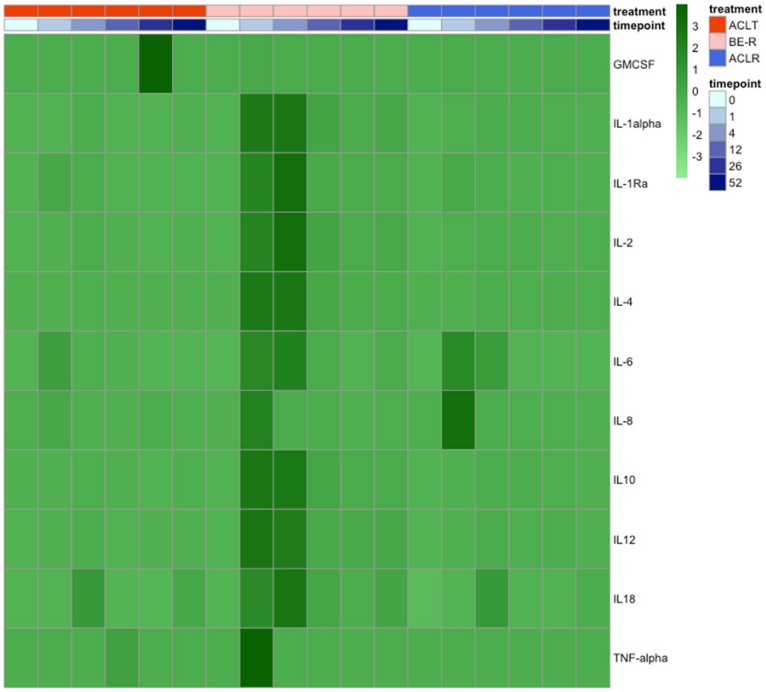

Figure S2. Z-scores of concentrations of cytokines in each surgical group are shown at 0, 1, 4, 12, 26, and 52 weeks. Each square represents the distance of a data point from the mean concentration of the row expressed in terms of the number of standard deviations. For example, a Z-score of -2 would indicate that the sample is 2 standard deviations below the mean concentration of the row. Each cytokine has a different range of concentrations, so similar colors in different rows do not indicate similar concentrations. Therefore, rows cannot be directly compared.

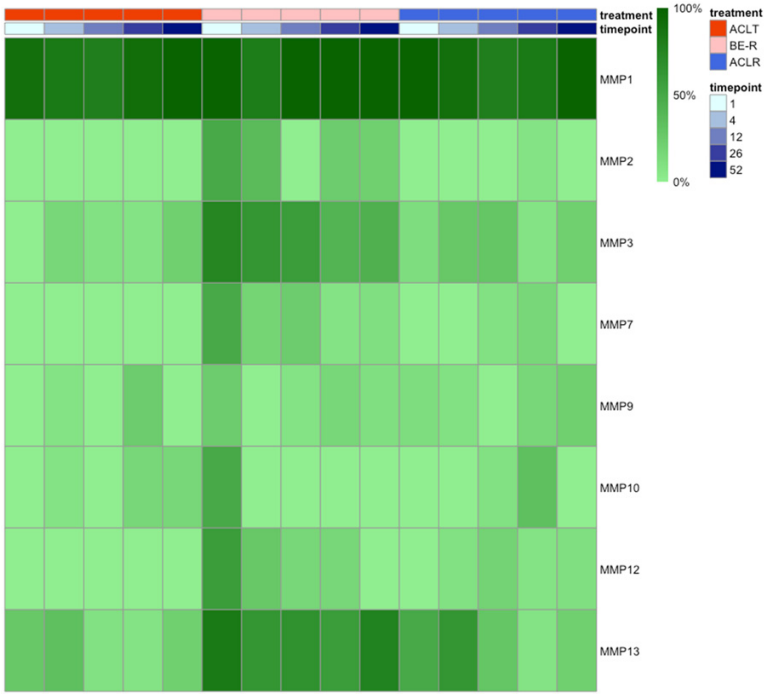

Figure S3. The percentages of synovial fluid samples with detectable levels of MMPs are shown in the heat map for each surgical group at 1, 4, 12, 26, and 52 weeks. Statistically significant comparisons are best interpreted with the data in Table S3.

Table S3. Differences in the proportion of samples that reach detectable levels of MMPs numerator is the number of animals with a detectable level of MMP at that time point and the denominator is the total number of animals where fluid was obtained

MMP	Time	ACLT	BE-R	ACLR	*P*-Value
MMP-1	Week 1	10/11 (90.9)	12/12 (100)	8/8 (100)	0.61
	Week 4	10/12 (83.3)	9/11 (81.8)	10/11 (90.9)	0.99
	Week 12	8/10 (80.0)	12/12 (100)	8/10 (80.0)	0.23
	Week 26	11/12 (91.7)	12/12 (100)	10/12 (83.3)	0.76
	Week 52	9/9 (100)	9/9 (100)	9/9 (100)	N/A
MMP-2	Week 1	0/11 (0.0)	6/12 (50.0)[#,+]	0/8 (0.0)	0.004
	Week 4	0/12 (0.0)	4/11 (36.4)[#,+]	0/11 (0.0)	0.01
	Week 12	0/10 (0.0)	0/12 (0.0)	0/10 (0.0)	N/A
	Week 26	0/12 (0.0)	3/12 (25.0)	1/12 (8.3)	0.29
	Week 52	0/9 (0.0)	2/9 (22.2)	0/9 (0.0)	0.31
MMP-3	Week 1	0/11 (0.0)	9/12 (75.0)[#,+]	1/8 (12.5)	>0.001
	Week 4	2/12 (16.7)	7/11 (63.6)[#]	3/11 (27.3)	0.06
	Week 12	1/10 (10.0)	7/12 (58.3)[#]	3/10 (30.0)	0.06
	Week 26	1/12 (8.3)	5/12 (41.7)	1/12 (8.3)	0.17
	Week 52	2/9 (22.2)	4/9 (44.4)	2/9 (22.2)	0.66
MMP-7	Week 1	0/11 (0.0)	6/12 (50.0)[#,+]	0/8 (0.0)	0.004
	Week 4	0/12 (0.0)	2/11 (18.2)	0/11 (0.0)	0.20
	Week 12	0/10 (0.0)	3/12 (25.0)	1/10 (10.0)	0.35
	Week 26	0/12 (0.0)	1/12 (8.3)	2/12 (16.7)	0.76
	Week 52	0/9 (0.0)	1/9 (11.1)	0/9 (0.0)	0.99
MMP-9	Week 1	0/11 (0.0)	3/12 (25.0)	1/8 (12.5)	0.22
	Week 4	1/12 (8.3)	0/11 (0.0)	1/11 (9.1)	0.99

	Week 12	0/10 (0.0)	1/12 (8.3)	0/10 (0.0)	0.99
	Week 26	3/12 (25.0)	2/12 (16.7)	2/12 (16.7)	0.99
	Week 52	0/9 (0.0)	1/9 (11.1)	2/9 (22.2)	0.75
MMP-10	Week 1	0/11 (0.0)	5/10 (50.0)[#,+]	0/8 (0.0)	0.003
	Week 4	1/12 (8.3)	0/11 (0.0)	0/10 (0.0)	0.99
	Week 12	0/10 (0.0)	0/12 (0.0)	1/10 (10.0)	0.63
	Week 26	1/6 (16.7)	0/5 (0.0)	1/3 (33.3)	0.67
	Week 52	1/6 (16.7)	0/8 (0.0)	0/7 (0.0)	0.28
MMP-12	Week 1	0/11 (0.0)	7/12 (58.3)[#,+]	0/8 (0.0)	0.0007
	Week 4	0/12 (0.0)	3/11 (27.3)	1/11 (9.1)	0.10
	Week 12	0/10 (0.0)	2/12 (16.7)	2/10 (20.0)	0.52
	Week 26	0/12 (0.0)	2/12 (16.7)	1/12 (8.3)	0.76
	Week 52	0/9 (0.0)	0/9 (0.0)	1/9 (11.1)	0.99
MMP-13	Week 1	3/11 (27.3)	10/12 (83.3)[#]	4/8 (50.0)	0.03
	Week 4	4/12 (33.3)	7/11 (63.6)	7/11 (63.6)	0.27
	Week 12	1/10 (10.0)	8/12 (66.7)[#]	3/10 (30.0)	0.02
	Week 26	1/12 (8.3)	7/12 (58.3)[#,+]	1/12 (8.3)	0.007
	Week 52	2/9 (22.2)	7/9 (77.8)[#,+]	2/9 (22.2)	0.03

P values in the table are for the overall effect of group based on the GEE analysis; [#]post-hoc analysis with Holm test adjusted *p*-value demonstrated significant difference in comparison to the ACL-T group with P<0.05; [+]post-hoc analysis with the adjusted *p*-value demonstrated significant difference in comparison to the ACLR group.

Table S4. Concentrations of MMPs (mean ± SD, pg/ml) with the minimum detectable concentration set to zero and number of surgical joints (N) involved

MMPs	Time	ACLT	BE-R	ACLR
MMP-1	Preop	43.81±58.08 (8)	39.56±27.25 (9)	23.74±12.33 (10)
	1 week	230.27±110.08 (11)	199.2±82.85 (12)	332.07±130.34 (8)
	4 weeks	198.11±113.64 (12)	241.69±178.63 (11)	305.84±115.29 (11)
	12 weeks	213.5±142.73 (10)	181.33±102.8 (12)	167.11±116.81 (10)
	26 weeks	97.8±60.4 (12)	110.61±38.69 (12)	109.18±60.25 (12)
	52 weeks	111.31±34.4 (9)	135.93±43.93 (9)	94.57±32.27 (9)
MMP-2	Preop	0±0 (8)	0±0 (9)	0±0 (10)
	1 week	0±0 (11)	355.86±540.04 (12)	0±0 (8)
	4 weeks	0±0 (12)	237.54±663.09 (11)	0±0 (11)
	12 weeks	0±0 (10)	0±0 (12)	0±0 (10)
	26 weeks	0±0 (12)	20.01±58.16 (12)	6.43±22.26 (12)
	52 weeks	0±0 (9)	27.8±55.16 (9)	0±0 (9)
MMP-3	Preop	0.01±0 (8)	0.01±0 (9)	19.66±62.13 (10)
	1 week	0.01±0 (11)	144.29±113.68 (12)	7.62±21.52 (8)
	4 weeks	12.5±34.07 (12)	83.35±71.78 (11)	30.26±52.4 (11)
	12 weeks	4.2±13.26 (10)	68.48±89.26 (12)	26.44±47.2 (10)
	26 weeks	8.25±28.56 (12)	35.56±47.48 (12)	10.94±37.85 (12)
	52 weeks	23.52±52.12 (9)	57.92±74.71 (9)	21.89±48.62 (9)
MMP-7	Preop	0±0 (8)	0±0 (9)	0±0 (10)
	1 week	0±0 (11)	251.29±339.66 (12)	0±0 (8)
	4 weeks	0±0 (12)	190.25±583.53 (11)	0±0 (11)
	12 weeks	0±0 (10)	97.05±209.04 (12)	13±41.11 (10)
	26 weeks	0±0 (12)	31.34±108.58 (12)	22.95±72.2 (12)
	52 weeks	0±0 (9)	24.78±74.35 (9)	0±0 (9)
MMP-9	Preop	0±0 (8)	0±0 (9)	1.65±5.2 (10)

	1 week	0±0 (11)	2.96±6.97 (12)	0.51±1.44 (8)
	4 weeks	1.75±6.08 (12)	0±0 (11)	0.43±1.43 (11)
	12 weeks	0±0 (10)	1.66±5.73 (12)	0±0 (10)
	26 weeks	5.55±17.71 (12)	1.36±3.23 (12)	1.1±3.01 (12)
	52 weeks	0±0 (9)	11.97±35.91 (9)	1.41±2.97 (9)
MMP-10	Preop	11.97±8.04 (8)	6.57±5.88 (9)	6.31±6.94 (10)
	1 week	0±0 (11)	8.73±10.76 (12)	0±0 (8)
	4 weeks	0.86±2.96 (12)	0±0 (11)	0.44±1.44 (11)
	12 weeks	0±0 (10)	0±0 (12)	2.54±8.03 (10)
	26 weeks	3.27±4.1 (12)	1.83±1.95 (12)	5.93±11.45 (12)
	52 weeks	4.02±4.78 (9)	0.61±1.83 (9)	1.45±2.91 (9)
MMP-12	Preop	3.01±8.51 (8)	0±0 (9)	0±0 (10)
	1 week	0±0 (11)	46.15±54.48 (12)	0±0 (8)
	4 weeks	10.39±36 (12)	9.93±17.42 (11)	2.68±8.88 (11)
	12 weeks	0±0 (10)	8.43±22.06 (12)	7.47±15.83 (10)
	26 weeks	0±0 (12)	0.69±1.7 (12)	0.09±0.31 (12)
	52 weeks	0±0 (9)	0±0 (9)	1.45±2.91 (9)
MMP-13	Preop	26.63±75.29 (8)	41.88±89.82 (9)	44.03±108.14 (10)
	1 week	14.11±29.52 (11)	972.58±892.68 (12)	90.64±163.43 (8)
	4 weeks	74.98±126.16 (12)	455.48±490.49 (11)	183.78±197.09 (11)
	12 weeks	16.44±51.94 (10)	241.17±275.23 (12)	109.59±191.74 (10)
	26 weeks	16.62±57.55 (12)	100.35±152.3 (12)	31.84±110.27 (12)
	52 weeks	37.51±82.22 (9)	123.37±144.6 (9)	31.7±77.38 (9)

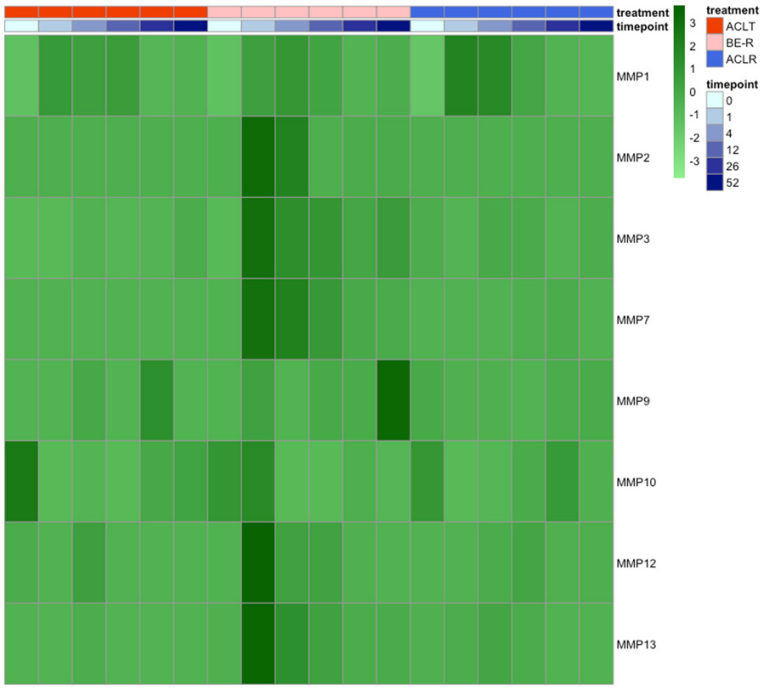

Figure S4. Z-scores of concentrations of MMPs in each surgical group are shown at 0, 1, 4, 12, 26, and 52 weeks. Each square represents the distance of a data point from the mean concentration of the row expressed in terms of the number of standard deviations. Each MMP has a different range of concentrations, so similar colors in different rows do not indicate similar concentrations. Therefore, rows cannot be directly compared.