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14. ABSTRACT: Since it is known that cartilage catabolic enzymes in synovial fluid (SF) play a critical role in the onset and progression of post traumatic osteoarthritis (PTOA), the objective of this study is to demonstrate that intra-articular injection of α -2 Macroglobulin (A2M), a master inhibitor of these enzymes, would serve as an early therapeutic agent to prevent PTOA after joint injury. In this proposed study, we will test whether early supplemental intra-articular A2M injections will attenuate PTOA pathogenesis 15 weeks after joint injury in the mini-pig model in vivo and we will explore the mechanism how A2M prevents PTOA via blockage IL-1/NF-kb pathway in vitro. In year 3, we published a peer review paper entitled "A2M Inhibits Chondrocyte Catabolism by Blocking IL-1 β /NF- κ B Pathway" on JOR. In year 4, we have collected all slides from 48 pigs and analyzed these samples. Our second paper entitled "A Novel Large Animal Model of Posttraumatic Osteoarthritis Induced by Inflammation with Mechanical Stability" just published on AJTR. Our third paper was published on Biomolecules entitled "The Effects of an Osteoarthritic Joint Environment on ACL Damage and Degeneration: A Yucatan Miniature Pig Model". The fourth paper just submitted to AJSM entitled " α -2-Macroglobulin Attenuates Posttraumatic Osteoarthritis Cartilage Damage by Inhibiting Inflammatory Pathways in a Preclinical Yucatan Minipig Model". Successful implementation of an injectable therapeutic to prevent the PTOA has the potential to significantly improve the quality of life for thousands of wounded warriors, as well as maximizing their function for return to duty or civilian life. We have achieved the goals we proposed in the study successfully.					
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TABLE OF CONTENTS

	<u>Page</u>
1. Introduction	3
2. Keywords	3
3. Accomplishments	3-4
4. Impact	4
5. Changes/Problems	4-5
6. Products	5
7. Participants & Other Collaborating Organizations	5
8. Special Reporting Requirements	5
9. Appendices 1-10	6-18

1. **Introduction:** Since it is known that cartilage catabolic enzymes in synovial fluid (SF) play a critical role in the onset and progression of post traumatic osteoarthritis (PTOA), the objective of this study is to demonstrate that intra-articular injection of α -2 Macroglobulin (A2M), a master inhibitor of these enzymes, would serve as an early therapeutic agent to prevent PTOA after joint injury. The mechanisms of PTOA following joint injury are likely due to the biological insults at the time of injury. Anterior cruciate ligament (ACL) injury is one of the most common injuries in the young and military population and is known to place the patient at risk for PTOA. Traditionally, these injuries are surgically treated with ACL reconstruction (ACL-R) to restore biomechanical stability to the joint. However, even with our best current surgical techniques, these patients remain at high risk for PTOA. The dramatic increase in SF catabolic enzymes following joint injury initiates PTOA. Therefore, early intervention to eliminate catabolic enzymes may be critical for the prevention of PTOA. In this study, we will test **1)** limiting catabolic enzymes through the intra-articular injection of A2M immediately following injury will reduce the microscopic cartilage integrity score (OARSI) and catabolic enzyme concentrations in the A2M treated animals compared to the no-A2M treated control; **2)** the vertical ground reaction forces between limbs will return to pre-operative values early in the injury A2M treated animals compared with injury (no A2M) treated animals; **3)** we will explore the mechanism in which how A2M prevents PTOA using cell culture. Successful implementation of an injectable therapeutic to prevent the PTOA has the potential to significantly improve the quality of life for thousands of wounded warriors, as well as maximizing their function for return to duty or civilian life.
2. **Keywords:** Post traumatic osteoarthritis (PTOA); cartilage; joint injury; inflammation; therapy; α -2 Macroglobulin (A2M); knee joint; Anterior cruciate ligament (ACL)
3. **Accomplishments:** we have achieved the goals we proposed in the study successfully. See the detail below.
 - 3-1. **Major goals of the project.** We have three goals for this project: **1)** limiting catabolic enzymes through the intra-articular injection of A2M immediately following injury will reduce the microscopic cartilage integrity score (OARSI) and catabolic enzyme concentrations in the A2M treated animals compared to the no-A2M treated control; **2)** the vertical ground reaction forces between limbs will return to pre-operative values early in the injury A2M treated animals compared with injury (no A2M) treated animals; **3)** we will explore the mechanism in which how A2M prevents PTOA using cell culture.
 - 3-2. **Accomplishments. 3-2-1.** We have been practicing the surgical procedure in order to success perform the surgery using pig carcass specimen collected from residence clinical training (N=10). We also did synovial fluid collection training (N=2) using the pig knee specimen. **3-2-2 (major goal 1).** In

vivo study, we have performed 48 live pigs' surgery with A2M treatment and collected all knee joint samples at the end of February 2022. The knee samples from 48 pigs have been sent for section and staining. We have analyzed these slides collected from all the 48 pigs. **3-2-3 (major goal 2)**. We have collected and analyzed the gait data from the 48 pigs. We have analyzed the gait data. **3-2-4 (major goal 3)**. In vitro study, we isolated chondrocytes from pig knee (N=2) for the mechanism study. We also used human chondrocyte cell line (C28) to validate the pig cell result because the human chondrocytes are more relevant to human osteoarthritis. We found that the antibody IL-1 β (cell signaling) interacted with IL-1 β protein in both pig chondrocytes and human chondrocytes, but the NF- κ B antibody (cell signaling) only interacted with NF- κ B protein in the human chondrocytes and did not interact with pig chondrocytes. We searched the internet and could not find any NF- κ B antibody that can interact with pig chondrocytes. NF- κ B is a key target for the mechanism study. Therefore, we decide using human chondrocytes for the mechanism study. We found that In C28 cell line, A2M labeled with VivoTag™ 680 was easy detected by fluorescence microscopy, which indicated that A2M was able to enter the cells (Figure 1). Western blotting and IP experiments further showed that A2M was able to bind to IL-1 β and inhibited IL-1 β (Figure 2) and NF- κ B in a dose dependent manner (Figure 3). IL-1 β induced the expression of NF- κ B, while A2M reduced the level of NF- κ B induced by IL-1 β . These findings indicated that A2M inhibits inflammation via binding to and inhibiting IL-1 β , which blocked NF- κ B pathway in C28 human chondrocytes. **3-2-5**. One abstract entitled "Alpha2-macroglobulin inhibits chondrocyte catabolism by blocking IL-1 β /NF κ B pathway" was presented in 2021 ORS (appendix 1). Two abstracts entitled "Alpha2-macroglobulin inhibits chondrocyte catabolism by blocking IL-1 β /NF κ B pathway" and "A Novel Mechanically Stable PTOA Model Of Inflammation: Swine Pilot Study Of Drilling Adjacent ACL Attachment" have been presented on ORS 2022 respectively (appendix 2 and 3). One manuscript entitled "A2M inhibits chondrocyte catabolism by blocking IL-1 β pathway" has been published at JOR (appendix 4). **3-2-6**. One poster and one oral presentation were presented in 2023 ORS (appendix 5 and 6). The second manuscript was published by AJTR (May-7-2023). (appendix 7). **3-2-7**. Two posters were presented in 2024 ORS (appendix 8 and 9). The third manuscript was published by Biomolecules (May-7-2023). (appendix 10). Our fourth manuscript was submitted to AJSM (appendix 11).

3-3. **Training and professional development.** Nothing to report.

3-4. **The results disseminated to communities of interest.** Nothing to report.

3-5. **Plan to do during the next reporting period.** This is a final report. We have finished all objectives as we proposed in the grant. We wish to explore the change of the subchondral bone if I have change in future.

4. Impact:

4-1. **Impact on the development of the principal disciplined of the project.** Nothing to report.

4-2. **Impact on other disciplines.** Nothing to report.

4-3. **Impact on technology transfer.** Nothing to report.

4-4. **Impact on society beyond science and technology.** Nothing to report.

5. Changes/problems:

5-1. **Changes in approach and reasons for changes.** We requested to modify the surgical procedure originally proposed in the grant to improve model performance and animal welfare. The request has been approved by both Brown IACUC and ACURO. The reasons for the request were: In getting ready to begin the study, my co-investigators, Drs Owens, Beveridge and Fleming, and I have been practicing the surgical procedure to core out the femoral insertion of the ACL in cadavers. The coring procedure was originally developed to represent an ideal ACL graft as the surgical trauma is induced while minimizing damage to the ACL (O'Brien et al: J Orthop Res 2013). In doing these practice trials, we found that the coring out procedure induced additional damage to the joint, such as cutting some of the fibers of the normal ACL and introducing cartilage damage. In brainstorming better ways to improve the model, we came up with a modification to the coring procedure originally described in the grant. We propose drilling two 2mm osseous tunnels through the tibia and femur that are adjacent to the intra-articular tibial and femoral insertions of the ACL. The modified procedure will still simulate the effects of the drilling required to implant a graft, which biologically induces cartilage damage, without the risk of mechanically damaging the ACL or articular cartilage. Unlike the coring model, which is only performed at the femoral insertion, we would perform the modified procedure on both the tibia and femur, which is

more representative of the drilling required during ACL reconstruction surgery. Furthermore, the drilling model has been previously used to surgically induce post-traumatic osteoarthritis in similar animal models (Huebner et al: J Orthop Res 2013) so we know it will have the same effect. As the modified procedure will induce less damage to the joint and take less OR time, it will be easier on the animals. Furthermore, we know that with the original coring procedure, there are times when the insertion will be violated so that the animal will need to be excluded from the study. Thus, from an animal welfare perspective, the new procedure will be optimal.

Aim 2 is to measure gait changes between the A2M-treated animals and the sham treated animals before surgery and at 15 weeks after surgery. Just prior to running the first group of 8 animals, we learned that the gait walkway had gotten wet when we were calibrating the system. Due to the water damage, the walkway is not functioning. While this problem most likely can be fixed, the manufacturer is requiring us to ship it back to the company for analysis and repair. This may take a few weeks. Unfortunately, the animals are in house and scheduled for surgery starting on the 27th of August. However, the cost of housing the animals while waiting for the walkway to be repaired would be too much for the budget. Thus, we are proposing to not measure the gait pre-operatively in the first 8 animals of the study. I do not think this will be a serious concern as we will have the system up and running to get the more important 15 week post-operative assessment, in which we will still be able to make comparison of the gait parameters between the A2M and sham treated animals at that time point. We intend to perform the pre- and post-operative measures on the subsequent groups of animals for this study.

In the project, our priority goal is to inhibit the catabolic enzymes by A2M in synovial fluid (SF) because the SF is a main nutrition source for cartilage. However, the inhibition of the catabolic enzymes presented in cartilage might have some benefits. Therefore, we proposed adding lipid nanoparticle (LNP) delivery system that may help A2M diffusing into cartilage to inhibiting the catabolic enzymes presented in cartilage. The LNP was provided by Dr. Ge Zhang from Hong Kong Baptist University in our previous study. Unfortunately, they can't provide LNP for our large animal study. Therefore, the approved IACUC and ACURO don't include LNP delivery system. It should be noted that the modified procedure will not change the overall experimental design or the cost of the study.

5-2. Actual or anticipated problems or delays and actions or plans to resolve them. As we reported in the second year report, the project may delay due to the pandemic. Whole brown labs were reopened in July 2020. We have collected all samples and send the samples out for the section and staining by the end of March in year 2022. We got the section back by the end of the June 2022. We have analyzed the sections.

To today, we have finished the aim-1, aim-2, and aim-3 as we proposed in the DoD grant. Three papers have been published and one is in the review process.

I notice that there was a high incidence of a pre-excited cartilage damage in the Yucatan mini-pig model from our study. I believe it is important to let the research know the information, especially in post-traumatic osteoarthritis (PTOA) filed. Currently, I am collecting and analyzing the data and will try to write a short report for publication before I retire (July 1st, 2024). However, I have one potential problem, i.e., I am not sure where I can get a financial support for publication from my department If the manuscript is accepted after I retire. I will retire on June 30, 2024.

5-3. Changes that had a significant impact on expenditures. N/A

5-4. Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents. Nothing to report.

6. **Products:** Nothing to report.

7. **Participants & other collaborating organization:**

7-1. Individual worked on the project (at least one person month per year)

Name	Project role	Research Identifier	Nearest person	Contribution to project	Funding
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		(e.g. ORCID ID)	month worked		support
Lei Wei	PI	https://orcid.org/0000-0003-0352-1517	6	Participate in the experiment and Monitor the process. Writing report	
Changqi Sun	Research associate	N/A	Terminated on 12/31/23	Perform experiment and collect data	

7-2. Has there been a change in the activity since the last reporting period? Nothing to report.

7-3. What other organizations were involved with the project as partners? Nothing to report. There was no other organization involved with the project.

8. Special reporting requirement. Nothing to report.

9. Appendices

Appendices (1)

One 2021 ORS abstract.

A2M Inhibits Chondrocyte Catabolism by Blocking IL-1 β /NF- κ B Pathway

Changqi Sun¹, Can Cao¹, Braden C. Fleming¹, Brett D. Owens¹, Jillian E. Beveridge², Scott Mcallister¹, Lei Wei¹

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Disclosures: BCF is a founder of MIACH Orthopaedics, receive royalties from Springer Publishing, and receives a stipend from the American Journal of Sports Medicine. BDO receives royalties from conmed and stock from options vivorte.

INTRODUCTION: Dramatic increases in various inflammatory enzymes have been previously correlated with cartilage degeneration, a hallmark of osteoarthritis (OA). Specifically, IL-1 β , a master proinflammatory cytokine, is implicated as an important player in the development of post-traumatic osteoarthritis (PTOA) through upregulation of NF- κ B, which activates catabolic enzymes that may mediate the cartilage degradation seen in early PTOA. Alpha 2-macroglobulin (A2M) is major anti-inflammatory cytokine that can inhibit IL-1 β and its downstream effects. In recent studies, A2M was shown to reduce catabolism-associated cartilage damage in vitro and in preclinical PTOA models, thus identifying it as a potential therapeutic agent. However, the mechanism of action of A2M in slowing PTOA pathogenesis was unclear. We hypothesized that A2M binds and neutralizes IL-1 β , blocking the downstream NF- κ B-induced catabolism seen in PTOA joints. The objective of this study was to test our hypothesis using human (C28) and pig chondrocytes in vitro.

METHODS: Human (cell line C28) and pig chondrocytes were incubated with A2M protein (Sigma)-labeled with VivoTagTM 680 (0.25 mg/ml). Additional chondrocytes were incubated with VivoTagTM 680 alone as a control. All chondrocytes were then treated with IL-1 β (8 ng/mL)(Cell signaling) and incubated for 2 days. The location of VivoTagTM 680-A2M post-incubation was detected by confocal fluorescence microscopy. The degree of binding between A2M and IL-1 β was evaluated through immunoprecipitation (IP) using the IL-1 β antibody (Cell signaling). Catabolic proteins, including IL-1 β and NF κ B pathway products, were quantified by Western blot.

RESULTS: A2M labeled with VivoTagTM 680 was easily detected in the cytoplasm of C28 human and pig chondrocytes by fluorescence microscopy (Figure 1). IP experiments demonstrated that A2M was able to bind IL-1 β . Western blotting revealed that IL-1 β upregulated C28 cell NF- κ B in a dose-dependent manner (Figure 2A and B), and that A2M dose-dependently reduces IL-1 β and NF- κ B (Figure 3). These findings indicate that A2M was able to enter chondrocytes and bind IL-1 β , which led to reduced NF- κ B expression. Thus, A2M was shown to neutralize IL-1 β and to reduce the NF κ B pathway-linked activation of proinflammatory responses. A2M also inhibited the level of IL-1 β in pig chondrocytes, but due to the unavailability of pig-specific antibodies, we were unable to obtain NF- κ B data from pig chondrocyte experiments (Figure 2C) .

DISCUSSION: Our findings advance our understanding of the complex anti-inflammatory mechanism of A2M in attenuating the pro-inflammatory responses seen in PTOA development. The results of this study confirm that A2M inhibits the IL-1 β /NF- κ B signaling pathway in human chondrocytes in vitro, which likely plays a critical role in cartilage degradation, especially in early PTOA. Thus,

A2M may be a viable therapy to slow PTOA development in patients with traumatic joint injuries or joint surgeries resulting in significant inflammation. In vivo studies will be needed to confirm this finding.

SIGNIFICANCE/CLINICAL RELEVANCE: A2M inhibits catabolism by binding to IL-1 β and blocking the IL-1 β /NF- κ B signaling pathway. This finding may result in a novel PTOA-preventative therapy utilizing A2M to reduce inflammation-induced catabolic activity and protect cartilage after major joint injuries or surgeries. An example application could be supplemental intra-articular injection of A2M shortly after joint injury, which may in turn prevent the progression of PTOA.

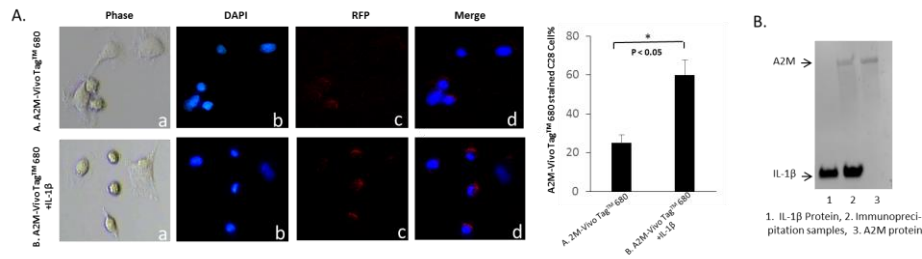


Fig.1. A2M entered into C28 chondrocytes as detected by immunofluorescence assay (A) and bound to IL-1 β as detected by IP (B).

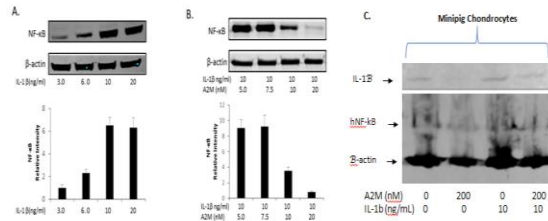


Fig.2. IL-1 β induced C28 chondrocyte NF- κ B expression in a dose-dependent manner (A). A2M inhibited the level of C28 cell NF- κ B induced by IL-1 β in a dose-dependent manner as detected by Western blotting (B). A2M inhibited the level of pig chondrocyte IL-1 β as detected by Western blotting (C).

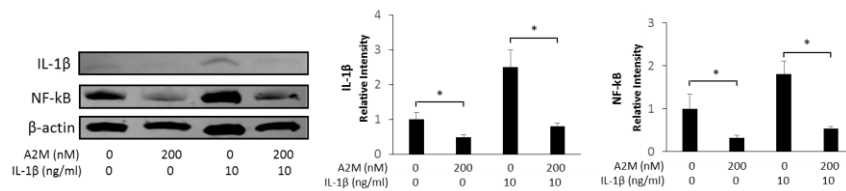


Fig.3. A2M treatment (200nM) reduced both the levels of IL-1 β and NF- κ B expression in C28 cells detected by Western blotting (left) and the quantitate data of IL-1 β and NF- κ B was showed (right).

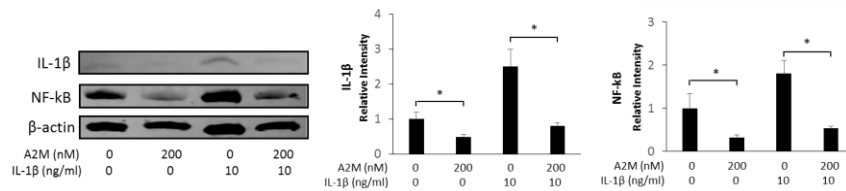


Fig.3. A2M treatment (200nM) reduced both the levels of IL-1 β and NF- κ B expression in C28 cells detected by Western blotting (left) and the quantitate data of IL-1 β and NF- κ B was showed (right).

Appendices (2)

2022 ORS abstract (1).

Cartilage Damage is Associated with Synovium Inflammation: A Novel Porcine Model of Post-traumatic Osteoarthritis

Changqi Sun¹, Kenny Chang¹, Braden C. Fleming¹, Brett D. Owens¹, Andrew Gage¹, Jillian E. Beveridge², Scott Mcallister¹, Meggin Costa¹, Megan Pinette¹, Ying Xiao¹, Lei Wei¹

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Disclosures: BCF is the founder of MIACH Orthopedics, receives royalties from Springer Publishing, and receives a stipend from the American Journal of Sports Medicine.

INTRODUCTION: Post-traumatic osteoarthritis (PTOA) is initiated by joint injury and affects various tissues such as articular cartilage, subchondral bone, and synovium. It has been noted that inflammatory factors are associated with functional changes of the articular cartilage, subchondral bone, and synovial membrane in osteoarthritis. However, it is unclear whether inflammation alone plays a similar role in PTOA pathogenesis. The synovium is thought to be the initial site of the inflammatory process, which if left untreated promotes irreversible damage in the adjacent cartilage and bone. To identify the role of inflammatory mediators of the synovial membrane in the pathogenesis of PTOA, we created a surgical model of PTOA using Yucatan minipigs. The surgical model entails drilling two osseous tunnels through the tibia and another two through the femur adjacent to the intra-articular tibial and femoral insertions of the ACL, respectively. The procedure simulates the effects of the drilling required to implant a graft in ACL reconstruction surgeries. The surgical model aims to biologically induce cartilage damage without mechanically damaging the ACL or articular cartilage. Thus, the model can be used to investigate the isolated effects of injury-induced inflammation without mechanical contributions from knee destabilization. Drilling into the non-load area alters neither loading of cartilage nor the mechanics of the joint. Our hypothesis was that an inflammatory synovial membrane promotes the pathogenesis of PTOA and is associated with cartilage destruction.

METHODS: Eight 15–16-month-old Yucatan minipigs weighing between 49–58kg were divided into a sham group and surgically-induced OA group (n=4 pigs/group). The animals were anesthetized and lateral arthrotomies were performed to gain access to the ACL of the left knee. Two osseous tunnels, 2mm in diameter and 15 mm deep, were drilled into the tibial bone adjacent to the anterior and posterior tibial insertions of the ACL in the OA group. The drilling procedure was repeated at the anterior medial and posterior lateral edges of the femoral insertion of the ACL. The sham animals underwent the same procedure but without drilling. Both left and right knee joints were harvested 15 weeks post-surgery. We sampled synovial tissue from the suprapatellar fold, which normally has an adipose areolar sub-synovial tissue so that the sub-intimal fibrosis could be scored. Paraffin cartilage sections were prepared for Safranin O-fast green staining. Frozen synovium sections were prepared for hematoxylin & eosin (H&E) staining. Cartilage integrity and synovium features (intimal hyperplasia and inflammatory cell infiltration) were evaluated using modified microscopic scoring systems (1). At least three randomly selected areas of the synovium were scored per section for each observation. Total RNA was isolated from the synovial membrane tissue using TRIzol Reagent (Invitrogen), and cDNA was synthesized with iScript cDNA synthesis kit (Bio-Rad). RT-PCR was used to evaluate secondary outcomes of IL-1 β , NF- κ B, MMP13, TNF- α inflammatory gene expression levels in the synovium.

RESULTS: Safranin O-fast green staining revealed significantly more cartilage degeneration of the medial tibial plateau (Fig.1) in the surgically-induced OA group compared to the sham group. H&E staining showed features of synovitis (multi-layer synovial membrane and inflammatory cells) in the surgical (left) knee of the surgically-induced OA group more than either the contralateral (right) knee or sham group (Fig.2). The synovial tissues in the surgically induced OA group had significantly higher mRNA expression levels of IL-1 β , NF- κ B, MMP13, TNF- α than those in the sham group (Fig.3).

DISCUSSION: Our porcine model demonstrates that synovium inflammation following isolated intra-articular bone injury is associated with intra-articular cartilage degeneration. Thus, this porcine model may be used to study the independent effects of synovium inflammatory mediators in the pathogenesis of osteoarthritis, especially in PTOA. The results of this study demonstrate that the increase of synovium inflammatory factors may be associated with cartilage damage in PTOA, which likely plays a critical role in the OA development. Our findings advance our understanding of synovium inflammation in the complex pathogenesis of osteoarthritis.

SIGNIFICANCE/CLINICAL RELEVANCE: Our porcine model successfully demonstrates that inflammation following isolated intra-articular bone injury can stimulate PTOA. The findings are helpful in elucidating the mechanism of cartilage degeneration and inflammatory mediators in the pathogenesis of PTOA. The model will be used to test a novel PTOA-preventative therapy that inhibit inflammation to protect cartilage after major joint injuries or surgeries.

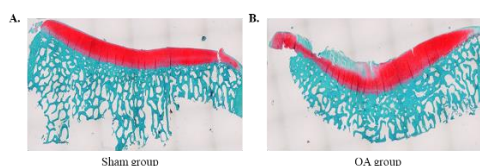


Fig.1. Drilling osseous tunnels induced cartilage degeneration at the tibia medial plateau.

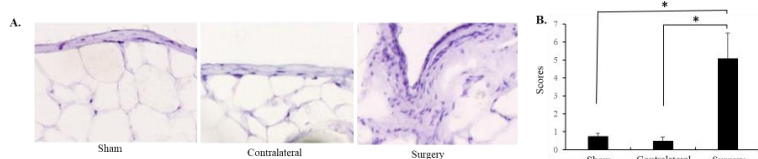


Fig.2. Drilling osseous tunnels induced synovitis.

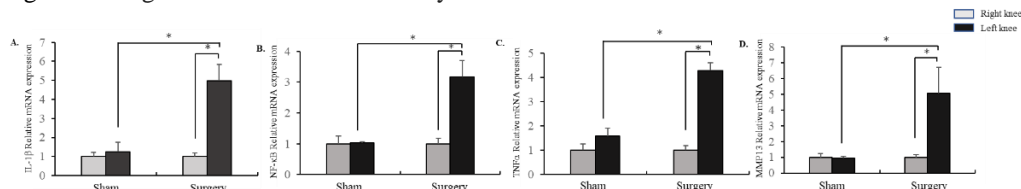


Fig.3. Increase of inflammatory mediators found in the surgery-induced synovium tissues compared with the contralateral knee.
Ref.1. Little et al. OA & Cartilage. 18(2010) S80-S9

Appendices (3)

2022 ORS abstract (2)

A Novel Mechanically Stable PTOA Model of Inflammation: Swine Pilot Study of Drilling Adjacent ACL Attachment

Kenny Chang¹ Changqi Sun, ¹Braden C. Fleming¹, Brett D. Owens¹, Andrew Gage¹, Jillian E. Beveridge², Scott Mcallister¹, Meggin Q. Costa¹, Megan P. Pinette¹, Ying Xiao¹, Lei Wei¹

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Disclosures: BCF is the founder of MIACH Orthopaedics, receives royalties from Springer Publishing, and receives a stipend from the American Journal of Sports Medicine.

Introduction: An “idealized” ACL reconstruction (IACLR) model involving a surgical procedure to core out the femoral insertion of the ACL was originally developed to study posttraumatic osteoarthritis (PTOA) following ACL reconstruction while minimizing direct damage to the ACL (1). However, while attempting to replicate the procedure on Yucatan minipigs, we found that the coring procedure induced additional damage to the ACL. To adapt the IACLR model to the pig, we proposed drilling two osseous tunnels 2mm in diameter through each of the tibia and femur adjacent to the intra-articular tibial and femoral ACL insertions. Unlike the coring model, which is only performed at the femoral insertion, the modified procedure is performed on both the tibia and femur, which is more representative of the drilling required during ACL reconstruction surgery. We hypothesize that the modified IACLR procedure (mIACLR) will mimic the effects of the drilling required to implant an ACL graft, which eventually leads to the development of PTOA. We propose that the procedure biologically induces cartilage damage without inducing mechanical instability and without the risk of mechanically damaging the ACL or articular cartilage.

Methods: The study was approved by the Institutional Animal Care and Use Committees of Brown University and Rhode Island Hospital and was performed to meet the ARRIVE guidelines. Fourteen Yucatan minipigs (15-16-months-old) were divided into a mIACLR surgical group (4M and 3F; 57±7.8kg) and a sham control group (4M and 3F; 56.9±6.8kg). The animals were anesthetized and a lateral arthrotomy was performed on the left hind knee to gain access to the ACL. For the mIACLR group, two osseous tunnels 2mm in diameter and 15mm deep were drilled into the tibial bone adjacent to the anterior and posterior tibial insertions of the ACL. The drilling procedure was then repeated at the anterior medial and posterior lateral edges of the femoral insertion of the ACL. The incision was then closed in layers. The sham group underwent the same procedure but without the drilling. The contralateral knees (right) were used as internal controls. Both hind knees were harvested 15 weeks post-surgery. Medial and lateral sites of the tibial plateau and femoral condyles were assessed for macroscopic and microscopic damage using the OARSI grading system for large animal models (2). IL-1β and NFκ-b expression was determined by immunohistochemistry. Load asymmetry during gait was recorded by a pressure-sensing walkway measurement system (Tekscan) before (week 0) and four times after surgery (weeks 4, 8, 12, and 15). The differential weight bearing between the left (surgical) versus right (control) hind limb was calculated across gait cycles and expressed as a ratio.

Results: Two pigs were excluded from the study: one had a pre-existing loose body in the knee joint, and the other had visible pre-existing cartilage damage. Macroscopic degenerative changes detected by India ink staining were found on the articular surfaces of the medial tibial plateau and medial femoral condyles of the mIACLR surgical knee (Fig. 1a and 1b). The lesions were centrally located on both the medial tibial plateau and medial femoral condyle. There was minimal damage on the lateral compartment of the joints for both the mIACLR and sham group. The median lesion areas were significantly higher in the mIACLR surgical knee compared with the sham group and the contralateral knee (Fig. 1b). Microscopic assessment showed significant differences in cartilage degeneration between the mIACLR group and the sham animals (Fig. 2). A strong positive staining of IL-1β and NFκ-b was detected in cartilage of the mIACLR surgical knee compared with the sham. No significant differences in limb asymmetry were found before surgery and at 4, 8, 12, and 15 weeks after surgery among the mIACLR and sham control animals (Fig. 3).

Discussion: The proposed surgical procedure of intra-articular bone drilling adjacent to the insertions of ACL was sufficient to elicit cartilage damage while eliminating potential confounding mechanical instability. The macroscopic and microscopic cartilage damage scores were associated with the elevated inflammation factors IL-1β and NFκ-b. The results of the study support the hypothesis that inflammation following intra-articular bone or joint injury can induce PTOA. The model may provide a useful tool to test the role of inflammation in PTOA and may open avenues to study developing therapies.

Significance/clinical relevance: This novel model of PTOA can separate the complex interaction of mechanical and inflammatory factors. Thus, we can test the role of inflammation and develop PTOA-preventative therapy by specifically reducing inflammation following joint injury.

Ref. 1. Heard et al. *JOR*. 2011;29:1185-92. 2. Little et al. *OA & Cartilage*. 18(2010) S80-S92.

Fig. 1. (A) Macroscopic changes detected by India ink staining in the surgery drilling animals. (B) Quantity data of the mean lesion areas.
Fig. 2. Microscopic degenerative change of the medial tibial plateau and medial femoral condyles of the surgically drilling knee.

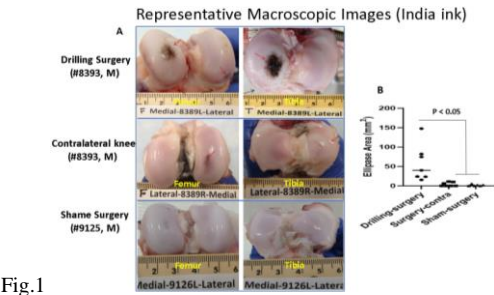


Fig.1

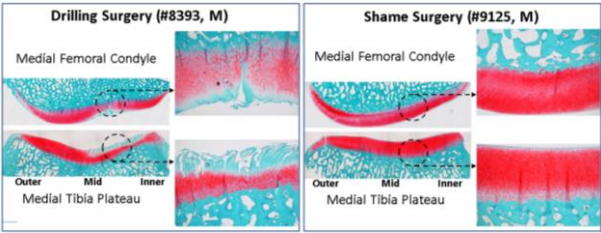
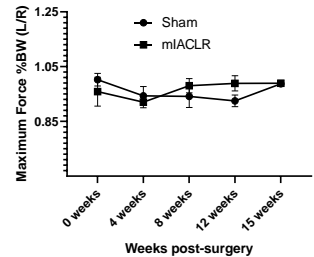


Fig.2.

Fig. 3. A strong positive staining of IL-1 β and NFk-b detected in the surgically drilling cartilage compared with the sham control. Fig. 4. No significant differences in joint kinematic change before surgery and post-surgery among these drilling and the sham control animals.



Appendices (4)

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A2M Inhibits Chondrocyte Catabolism by Blocking IL-1 β /NF- κ B Pathway

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INTRODUCTION: Dramatic increases in inflammatory enzymes have been previously correlated with cartilage degeneration, a hallmark of osteoarthritis (OA). Specifically, IL-1 β , a master proinflammatory cytokine, is implicated as an important player in the development of posttraumatic osteoarthritis (PTOA) through the upregulation of NF- κ B, which activates catabolic enzymes that may mediate the cartilage degradation. Alpha 2-macroglobulin (A2M) is major anti-inflammatory cytokine that can inhibit IL-1 β and its downstream effects. In recent studies, A2M was shown to reduce cartilage catabolism in vitro and in preclinical PTOA models, thus identifying it as a potential therapeutic agent. However, the mechanism of action of A2M is unclear. We hypothesized that A2M binds and neutralizes IL-1 β , blocking the downstream NF- κ B-induced catabolism seen in PTOA joints. The objective of this study was to test our hypothesis using human chondrocytes (C28) in vitro.

METHODS: Human (cell line C28) chondrocytes were incubated with the A2M protein (Sigma)-labeled with VivoTagTM 680 (0.25 mg/ml). Additional chondrocytes were incubated with VivoTagTM 680 alone as a control. All chondrocytes were then treated with IL-1 β (8 ng/mL)(Cell signaling) and incubated for 2 days. The location of VivoTagTM 680-A2M post-incubation was detected by confocal fluorescence microscopy. The degree of binding between A2M and IL-1 β was evaluated through immunoprecipitation (IP) using the IL-1 β antibody (Cell signaling). Catabolic proteins, including IL-1 β and NF- κ B, were detected by Western blot. Pro-inflammatory and chondrocyte-related gene expression were examined by qRT-PCR.

RESULTS: A2M labeled with VivoTagTM 680 was detected in the cytoplasm of C28 human chondrocytes by fluorescence microscopy. IP experiments demonstrated that A2M was bound to IL-1 β . Western blotting revealed that IL-1 β upregulated C28 cell NF- κ B in a dose-dependent manner, and that A2M dose-dependently reduced IL-1 β and NF- κ B. RT-PCR results showed that MMPs and TNF α decreased while expression of cartilage protective genes Col2, Type2, Smad4 and aggrecan increased. Thus, A2M was shown to neutralize IL-1 β and to reduce the NF κ B pathway-linked activation of proinflammatory responses.

CONCLUSION: A2M was shown to neutralize IL-1 β and to reduce the NF κ B pathway-linked activation of proinflammatory responses. This finding supports the development of a novel PTOA-preventative therapy utilizing A2M to reduce inflammation-induced catabolic activity and protect cartilage after a joint injury.

Introduction

Osteoarthritis (OA) is an increasingly prevalent debilitating joint disease that is a leading cause of disability. The pathogenesis of OA is multifactorial and still under investigation [1-3]. Dramatic increases in various inflammatory enzymes and cytokines have been correlated with joint damage seen in early OA [3, 4], making inflammatory mechanisms a point of interest as a potential therapeutic target, particularly for the treatment of posttraumatic osteoarthritis (PTOA), which occurs following a joint injury.

Specifically, IL-1 β , a master proinflammatory cytokine, is implicated as an important player in the development of PTOA through upregulation of the NF- κ B pathway, which activates catabolic enzymes that may mediate the cartilage degradation seen in early PTOA [4, 5]. Previous research demonstrated that the elevated levels of catabolic enzymes induce chondrocyte death and cartilage matrix degeneration after joint injury [4-8].

Concentrations of these enzymes, such as plasmin, cathepsin B, L, S, and matrix metalloproteinase (MMP) -3, -9, -13, have been shown to rise rapidly with tissue damage in surgically induced OA models [9, 10]. Thus, a possible intervention strategy is to reduce the post-injury surge of these enzymes to alter the degree of initial damage seen in PTOA.

Although several inhibitors, such as anti-TNF α , have been tested in animal and human studies [11-15], only minor or partial chondroprotective effects have been reported with their use. This result may reflect how targeting specific effector molecules may not generate a sufficient global influence over the many catabolic factors involved in PTOA pathogenesis. Additionally, the presence of drug-neutralizing antibodies in vivo has been associated with lower efficacy and higher relapse rates in patients on these biologics [11, 16-19].

Therefore, broader suppression of many catabolic factors may be more efficacious than targeting individual enzymes in the prevention of PTOA. Recent findings suggest that α 2-Macroglobulin (A2M) may be a strong therapeutic candidate due to its broad-spectrum ability to block catabolic cytokines and enzymes.[20, 21]

A2M, a 750 KDa molecule and major protease inhibitor, is found in serum and mainly is produced by the liver. The inhibitor acts on all classes of endoproteases and could potentially be used to slow or halt PTOA by neutralizing catabolic cascades in affected cartilage.[22, 23] Previous studies have demonstrated that A2M can enter into cells and bind to numerous cytokines, including transforming growth factor-beta 1 (TGF-beta 1), TGF-beta 2, nerve growth factor-beta (NGF-beta), platelet derived growth factor-BB (PDGF-BB), tumor necrosis factor-alpha (TNF-alpha), and basic fibroblast growth factor (bFGF) [18, 19, 24-26]. Additionally, A2M has been shown to reduce catabolism-associated cartilage damage in vitro and in preclinical PTOA models [9, 27], providing evidence of its promise as a therapeutic agent. However, the overall mechanism of action of A2M in slowing PTOA pathogenesis remains unclear.

A potential mechanism may involve IL-1 β , which is seen in early PTOA to up-regulate catabolism in vitro and in vivo via the NF- κ B pathway.[24-27] Prior work has shown that A2M is able to inhibit the majority of catabolic factors involved in this pathway, including IL-1 β itself [9, 28]. To examine this mechanism, in vitro human (C28) chondrocytes were evaluated for the degree of NF- κ B pathway upregulation induced by IL-1 β and cellular responsiveness to A2M in this investigation. We hypothesized that (1) A2M primarily neutralizes IL-1 β , leading to subsequent downregulation of multiple catabolic pathways and that (2) A2M was capable of disrupting PTOA progression because it inflammatory signaling both extracellularly and within chondrocytes.

Material and methods

Cell culture and Immunofluorescence

The human chondrocyte cell line C28 was a gift from Dr. Goldring MB [29]. The cells were grown in Dulbecco's Modified Eagle Media Nutrient mixture, DMEM/F12 1:1 (cat# 11320-033, Gibco) supplemented with 10% fetal bovine serum (cat# 16000069, Invitrogen) and 1% Penicillin/Streptomycin (cat# 15140122, Invitrogen). A2M protein (cat# M6159, Sigma) labeled with VivoTagTM 680 was synthesized using the Pierce NHS-Rhodamine Antibody Labeling Kit (cat# 53031, Thermo Scientific). A2M was mixed with NHS-Rhodamine reagent and incubated for 1hr at room temperature protected from light. Then the A2M protein labeled with VivoTagTM 680 was purified by spin columns.

C28 human cells were seeded in 12-well plates with a density of 1×10^5 /well and grown to 80-90% confluency before being incubated with 200nM VivoTagTM 680 labeled A2M for two hours. The cells were then treated with 10 ng/mL IL-1 β (cat# 201-LB, R&D, MN) and further incubated for 2 days. Additional C28 chondrocytes were incubated with VivoTagTM 680 alone as a control. After incubation, the cells were washed with PBS three times and DMEM/F12 media with 1 μ l/ml nuclear staining reagent Hoechst fluorochrome (cat# 33342, Sigma, St. Louis, MO) was added for DAPI testing. The positively stained cells were observed by inverted fluorescent microscopy (Nikon Eclipse, TS100) and analyzed using SPOT imaging software (Version 5.1, SPOT Advanced).

Immunostaining for IL-1 β

C28 cells were seeded in an 8-well tissue culture treated glass slide (cat# 34108, Corning, NY) at a density of 8×10^4 /well. The cells were incubated for 2 hours with A2M protein labeled with VivoTagTM 680, then treated with IL-1 β and incubated for 2 days. After incubation, the cells were washed once with PBS and fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature. The fixed cells were washed three times with 0.1% Triton X-100 /PBS. The cells were then incubated with rabbit anti-IL-1 β antibody (1:500; cat# P420B, Invitrogen) overnight at 4°C and subsequently washed three times with PBS. The washed cells were reacted with fluorescent secondary antibody Alex Fluor Plus 488 (1:1000; cat# A32731, Invitrogen) at room temperature for 1 hour, followed by three washes with PBS. After the chamber was removed, the slides were mounted with coverslips using mounting medium with DAPI (cat# H-1500, Vector Lab, CA). The positive-staining cells were observed using inverted fluorescent microscopy (NI-SSR, Nikon) and analyzed using imaging software (NIS-Elements AR, version 5.1). Three views were taken per well (blue nuclear staining, red A2M staining and green IL-1 β staining) with a total of 12 views from four wells selected for marker testing. The percentage of a well staining for each marker was defined as the proportion of all cells in view positive for the nuclear stain.

Immunoprecipitation and Western Blot analysis

C28 cells were incubated with A2M for 2 hours and treated with IL-1 β as previously described. The cells were lysed using the Complete Lysis Kit (cat# 04719956001, Roche). Lysates were incubated overnight with beads

of protein A/G Plus-Agrose (cat# sc-2003, Santa Cruz) coated with anti-A2M antibody. For the Western blot analysis, 100 µg of isolated and dissociated protein was loaded onto 4% to 15% SDS-PAGE mini-protein TGX Precast gel (cat# 456-1084, Bio-Rad Lab) and then blotted on membranes for evaluation of obtained protein. The primary antibodies used were all mouse-derived and include anti-A2M (1 µg/ml; cat# sc-390544, Santa Cruz), anti-β actin (1:1000; cat# sc-5546, Santa Cruz), anti-IL-1β (1:500; cat# sc-7884, Santa Cruz) and anti-NF-κB (1:1000; cat# abc, Abcan). To identify reactive proteins, the fluorescent secondary antibody Donkey IR (1:1000; cat# 926-32214, Odyssey) was used and visualized by fluorescent detection (LI-COR, Odyssey).

Quantitative real-time RT-PCR for inflammatory- and chondrocyte-related gene analysis

The effect of A2M on inflammatory chondrocyte damage was analyzed via quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) according to the published protocol (iQ SYBR Green Supermix, Bio-Rad). After C28 cells were incubated and treated, total RNA was extracted using the RNeasy Mini Kit (cat# 74004, Qiagen). Gene expression was measured through two methods: first-strand cDNA synthesis using a reverse transcription kit (cat# 1708890, Bio-Rad) and qRT-PCR using the iQTM SYBR Green Supermix kit (cat# 170-8887, Bio-Rad) in a real-time PCR system (CFX Connect, Bio-Rad). A total of 1 µg of RNA was used for cDNA synthesis; priming was conducted at 25 °C for 5 minutes, reverse transcription was conducted at 40 °C for 20 minutes, and reverse transcriptase inactivation was done at 95 °C for one minute. For qRT-PCR testing GAPDH was used as an internal control. Human-specific primers were designed as specified below in Table 1. PCR was performed for 40 cycles after an initial denaturation step at 95 °C for 3 min. Each cycle involved denaturation for 15s at 95 °C, annealing for 60s at 58 °C and extension for 40s at 72 °C. The reaction was terminated at 70 °C after a 10-min extension. Three independent PCR experiments were performed to obtain the relative level of expression for each gene.

Statistical Methods

All procedures were repeated three times as independent experiments. The mean and standard deviation for each measured result was calculated and used for comparison in one-way analysis of variance (Bonferroni's multiple comparison test) where $p < 0.05$ was considered significant.

Results

To establish that A2M can enter human chondrocytes, C28 cells were treated with labeled A2M and evaluated under fluorescent microscopy. After incubation, labeled A2M (red) was identified within the chondrocytes, demonstrating cellular uptake of the molecule. Of note, the proportion of A2M detected intracellularly was as high as 57% in cultures where IL-1β was added compared to around 25% in the control (Figure 1A), implying the uptake of A2M is associated with the local presence of IL-1β. To determine if A2M directly binds IL-1β within cells, immunoprecipitation (IP) of intracellular contents using anti-A2M antibody was performed; subsequent western blot of the isolated precipitates identified mainly A2M and IL-1β with little other protein, confirming that A2M preferentially binds IL-1β within the chondrocytes (Figure 1B).

Double-labeling immunofluorescence analysis was conducted to evaluate the in vitro interaction between A2M and IL-1β. The labeled A2M (red) was detected as previously described, and IL-1β was visualized using fluorescent secondary antibody Alex Flior Plus 488 (green). Overall, 57% of chondrocytes were positive for A2M with Vivo TagTM (red) and 74% stained positive for IL-1β (green). Additionally, 51% of cells demonstrated orange fluorescence, which implies many cells contained A2M and IL-1β near each other (Figure 2). This overlapping color signaling thus further evidences the intracellular co-localization and binding of the two molecules implied by the IP results.

To evaluate the impact of IL-1β binding by A2M on downstream inflammatory pathways, the relationship between A2M, IL-1β and NF-κB in chondrocytes was evaluated through western blot experiments. First, IL-1β was demonstrated to induce NF-κB protein expression in a concentration-dependent manner, reaching maximal effect at 10 ng/mL (Figure 3A). Second, A2M was shown to inhibit NF-κB protein expression in a concentration-dependent manner; the highest A2M concentration tested (200 nM) resulted in the lowest detected NF-κB protein (Figure 3B). In further experiments, the addition of 200 nM of A2M reduced resultant IL-1β and NF-κB protein levels in both cultures without exogenous IL-1β and those treated with 10 ng/mL of IL-1β (Figure 4). Taken together, these findings imply that the A2M-mediated reduction of NF-κB expression is related to IL-1β, with the most straightforward mechanism being direct A2M binding of IL-1β to inhibit its downstream signaling.

Real-time PCR was conducted to determine if A2M inhibition of IL-1 β resulted in altered gene expression in chondrocytes, especially for genes known to play a role in PTOA pathogenesis. In cultures incubated with A2M, cellular expression of inflammatory genes implicated in PTOA catabolic damage including the MMPs and TNF α decreased while expression of cartilage protective genes Col2, Smad4 and aggrecan increased (Figure 5). This result thus demonstrates that A2M is indeed capable of changing IL-1 β signaling to reduce catabolism-associated cartilage damage in vitro, providing further evidence for the viability of A2M as a novel treatment for altering the natural progression of PTOA.

Discussion

Prior studies have shown that A2M has chondroprotective effects such as inhibiting ADAMTS-4,-5,-7,-12 [16, 17], reducing ligament stump resorption following ACL injury [25], and enhancing tendon-bone healing of ACL grafts by inhibiting MMP-13.[26] Recently, we found that A2M is a negative regulator for many cartilage catabolic enzymes both in vitro and in vivo and that high levels of A2M are associated with low serum levels of MMP-3 and -13.[9] Furthermore, supplemental exogenous A2M was shown to inhibit a majority of the catabolic cytokines and enzymes influencing cartilage MMPs, including IL-1 β , IL-8, TNF- α , GM-CSF, MMP-3, -9, and -13.[28] *In vivo* studies further demonstrate that supplemental intra-articular A2M injection can attenuate OA pathogenesis and reduce the level of MMP-13 in synovial fluid using the rat ACLT model.[9, 10] This finding was validated in a collagen II induced arthritis model [29], where A2M also consistently reduced degrading factors and inhibited arthritic inflammation. However, the mechanism for how A2M mediates these observations was unclear. To this end, we hypothesized that that A2M likely acted upstream in catabolic signaling pathways to produce these multiple effects.

The findings of this study support our hypothesis by demonstrating that A2M is able to inhibit of IL-1 β within chondrocytes and thus attenuate multiple downstream catabolic pathways including that of NF- κ B and the MMPs. This mechanism for A2M action also supports the use of A2M therapeutically. Previous work has shown that IL-1 β is prominently present early on in traumatic joint injuries, which are a major risk factor for the development of PTOA; IL-1 β levels in synovial fluid can be 10 times higher than in serum after acute knee ligament injury. [30, 31] Thus by inhibiting this inflammatory cytokine found early in joint injuries, A2M may be able to disrupt the initial pathogenesis of PTOA.

The primary limitation of this study is that all experiments were conducted in vitro. Thus, these findings may not capture the true mechanism or alternate mechanisms that occur in vivo. These experiments did not evaluate for duration of A2M effects or changes over time in catabolic signaling, which additionally limits direct applicability in vivo. Finally, one cell line was used for all experiments, which may impact the generalizability of the findings to all human chondrocytes.

Conclusions

OA is a common disease with a complex, likely multifactorial pathogenesis and development; given the limited understanding of the condition's natural history, current treatment options mainly provide symptom relief and generally do not halt progression to severe joint degeneration. A2M has emerged as a potential treatment able to halt the development of early cartilage damage into significant disease, though its exact mechanism of action is largely unknown. Here we demonstrate that a likely mechanism for A2M attenuation of cartilage damage is through direct binding of IL-1 β , a master pro-inflammatory cytokine that induces many catabolic pathways implicated in early PTOA. In vitro experiments showed that A2M can enter human C28 chondrocytes, bind IL-1 β both intracellularly and extracellularly, and downregulate IL-1 β inducible mediators of catabolic damage such as NF- κ B protein and mRNA encoding for pro-inflammatory factors like MMP-1, -3, and -13. The major limitation of this work is that because it was done in vitro, the generalizability of the described mechanism to in vivo systems remains to be seen. Thus, next steps include in vivo testing of A2M's effect on synovial catabolic factors and PTOA outcomes.

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Appendix 5 and 6.

One ORS poster and one oral presentations:

1. Changqi Sun; Kenny Chang; Braden Fleming; Brett Owens; Rachel Bruns; Jillian Beveridge; Scott Mcallister; Meggin Costa; Megan Pinette; Xiao Ying; Lei Wei. **A2m Reduces Synovial Inflammation In A Yucatan Mini-pig PTOA Model.** ORS 2023 Dallas TX. Feb 10-14, 2023 Podium
2. Kenny Chang; Changqi Sun; Braden Fleming; Brett D. Owens; Jillian Beveridge; Scott McAllister; Meggin Costa; Megan Pinette; Janine Molino; Lei Wei. **A₂-Macroglobulin Reduces Post-traumatic Osteoarthritis Cartilage Degeneration By Inhibiting Catabolic Pathways.** ORS 2023 Dallas TX. Feb 10-14, 2023 Poster.

Appendix 7.

One manuscript was published by AJTR in year 2023.

Changqi Sun, PhD,¹ Kenny Chang, BS,^{1*} Braden C. Fleming, PhD,¹ Brett D. Owens, MD,¹ Jillian E. Beveridge, PhD,² Andrew Gage, MD,¹ Rachel C. Talley-Bruns, MD,¹ Scott McAllister, BA,¹ Meggin Q. Costa, BA,¹ Megan P. Pinette, BS,¹ Madalyn Hague, BS,¹ Janine Molino, PhD,¹ Ying Xiao, BS,¹ Shaolei Lu, MD PhD,¹ **Lei Wei**, MD PhD¹ A Novel Large Animal Model of Posttraumatic Osteoarthritis Induced by Inflammation with Mechanical Stability. Am J Transl Res **2023**;15(7):4573-4586. PMID: 37560216. PMCID: PMC10408525

Appendix 8: Two abstracts presented on 2024 ORS

Changqi Sun; Kenny Chang; Braden C. Fleming; Brett D. Owens; Jillian E. Beveridge; Lei Wei. A2m Attenuates Cartilage Degeneration By Binding To And Blocking The Il-1r1 Cascade In A Large Preclinical Pig Model. ORS 2024 Long Beach, CA. Feb 3-6, 2024

Kenny Chang; Changqi Sun; Fei Zhang; Guoxuan Peng; Lei Wei. A₂-Macroglobulin Reduces ACL Degeneration In A Posttraumatic Osteoarthritis Environment In The Yucatan Minipig. ORS 2024 Long Beach, CA. Feb 3-6, 2024

Appendix 9: The third publication

The Effects of an Osteoarthritic Joint Environment on ACL Damage and Degeneration: A Yucatan Miniature Pig Model. Elias Schwartz, Kenny Chang, Changqi Sun, Fei Zhang, Guoxuan Peng, Brett Owens, **Lei Wei**. [Biomolecules](#). **2023** Sep; 13(9): 1416. doi: [10.3390/biom13091416](#). PMCID: PMC10526460. PMID: [37759816](#)

Appendix 10: The fourth manuscript was submitted to American Journal of Sport Medicine

Title: α_2 -Macroglobulin Attenuates Posttraumatic Osteoarthritis Cartilage Damage by Inhibiting Inflammatory Pathways in a Preclinical Yucatan Minipig Model

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