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TITLE: Bisphosphonates in the prevention of post-traumatic osteoarthritis.

PRINCIPAL INVESTIGATOR: Christopher Price (Partnering PI)

CONTRACTING ORGANIZATION: University of Delaware
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14. ABSTRACT The following report details the final accomplishments and products with regard to the tasks (partnering-PI C. Price's responsibility) for grant PR120788P1 (Contract number: W81XWH-13-1-0147), encompassing the final reporting period from 08/01/2016 to 07/30/2017, as well as all prior reporting periods. Early, Focal Changes in Cartilage Cellularity and Structure Following Surgically-Induced Meniscal Destabilization in the Mouse - Published in David, et al. 2017 J Ortho Res. - Histological analysis of mouse joints following DMM demonstrated rapid (within 3-day of injury), and focal (spatially localized) changes in both the cellularity (loss of chondrocytes) and the structural integrity of articular cartilage. The findings shed new light onto the early pathology and time-course of PTOA development following DMM and suggest that acute changes to a specific and localized population of chondrocytes may be critical in the initiation of long-term cartilage degeneration following joint injury, and represent a potential target for chondro-protective/anti-osteoarthritic therapy. Repeated Intra-Articular Injection of Zoledronic Acid Suppresses Cartilage Erosions Following Destabilization of the Medial Meniscus in Mice (David, et al. 2018 In Preparation) - The present study has shown that repeated i.a. injection of ZA into the murine knee following DMM provides cartilage protection by modulating the underlying processes of chondrocyte death, proliferation, and proteoglycan production in a spatiotemporal dependent manner. In addition to AC changes, ZA also appears to affect whole joint changes including changes in the menisci, ectopic bone, and osteophytes.					
15. SUBJECT TERMS Post-traumatic osteoarthritis; cartilage degeneration; DMM model; bisphosphonate; zoledronic acid; chondro-protection; cartilage therapy; intra-articular injection, anti-osteoarthritic					
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- INTRODUCTION:** This grant focuses on the investigation of a pharmacological treatment for “post-traumatic osteoarthritis” (PTOA). PTOA is a disease of cartilage degeneration that results from acute joint trauma (e.g., torn cartilage, dislocated joints, and meniscal or ligamentous damage) (1, 2), injuries that are common in military service (3, 4). Currently, no preventative or curative treatments exist for PTOA, and afflicted individuals are resigned to the gradual degeneration and loss-of-function of the joint with few therapeutic options other than costly and highly invasive late-stage surgical approaches, including micro-fracture surgery and joint replacement. In a proof-of-concept study we reported that repeated systemic administration of the FDA-approved bisphosphonate drug zoledronic acid (ZA) immediately following joint injury could suppress the development of PTOA in the DMM (destabilization of the medial meniscus) mouse model of PTOA (5). However, the strong, and potential deleterious impact of BPs on bone remodeling (6, 7) and their resultant side effects represents a barrier to their systemic use in the clinical treatment of PTOA. In this grant, we proposed and are testing the efficacy of targeted ZA delivery, through localized intra-articular (i.a.) injection, to prevent PTOA while minimizing adverse skeletal health effects. Furthermore, we are exploring the cellular and molecular mechanisms underlying ZA’s anti-osteoarthritic potential in order to develop therapeutic PTOA treatments that are more cartilage specific. This annual report details the portion (Aim 2: Determine the chondro-protective effect of locally delivered ZA using an animal model) of the grant being performed within the partnering-PI’s lab (Price). This sub-project involves the pre-clinical testing of i.a. ZA injection for treating PTOA within a murine model of surgically induced PTOA (the DMM model). The purpose of this sub-project is to evaluate the *in vivo* mechanisms by which PTOA progresses within the DMM model and to establish the efficacy and mechanisms of action of i.a. ZA in preventing PTOA. This sub-project is establishing these properties through the measurement of the structural, morphological, biochemical, molecular, and cellular properties of cartilage and bone in mice treated with i.a. ZA following the induction of injury via the DMM model. Since the *in situ* repair of degenerate cartilage is a challenging, and a yet unrealized task, the prevention of PTOA through the innovative i.a. delivery of ZA may provide a simple, effective, and lower-cost treatment for lessening the burden of this disease. Furthermore, the knowledge of the chondro-protective mechanisms of ZA that may help to identify additional molecular, cellular, and biochemical targets by which OA/PTOA may be treated in the future.
- KEYWORDS:** *Post-traumatic osteoarthritis; cartilage degeneration; DMM model; bisphosphonate; zoledronic acid; intra-articular injection; chondro-protection; cartilage therapy; anti-osteoarthritic.*

- ACCOMPLISHMENTS:**

- What were the major goals of the project?**

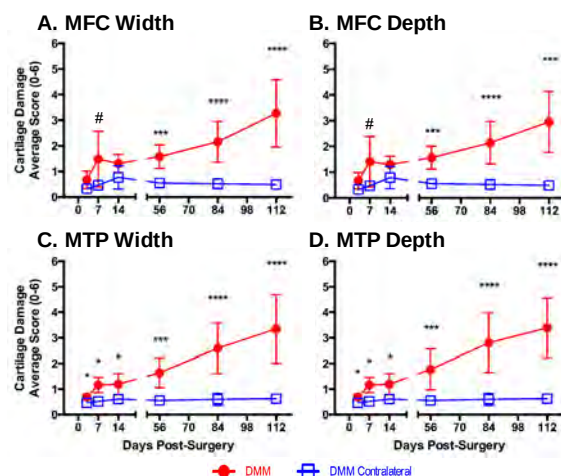
Specific Aim 1: Investigate the “in vivo chondro-protective effects of zoledronic acid (ZA) – a small-animal (Mouse) model of surgically induced PTOA and treatment”	Milestone achieved
Major Task 1: Evaluate the in vivo chondro-protective efficacy of immediate and delayed targeted local administration of zoledronic acid (ZA) to prevent post-traumatic osteoarthritis (PTOA)	
Subtask 1: Obtain final IACUC/ACURO animal protocol approval and finalize PTOA surgery and intra-articular (i.a.) injection procedure development and training	12/13
Subtask 2: Perform PTOA surgery (DMM and Sham), i.a. injections (ZA and vehicle; peri- & post-operative), and animal sacrifice/specimen harvesting; procedures will be performed in a batch-wise manner Animals Used: 383 Mice; Strain: C57BL/6J, [Jackson Labs]	3/16
Subtask 3: Micro-computed tomography (micro-CT) imaging-based characterization of joint bone structure following PTOA surgery and i.a. ZA intervention.	4/16
Subtask 4: Histological characterization/assessment of articular cartilage damage/protection following PTOA surgery and i.a. ZA intervention.	3/17
Subtask 5: Immunohistochemical evaluation/characterization of cartilage cellular responses following PTOA surgery and i.a. ZA intervention.	5/17
Major Task 2: Experiment Data Analysis, Yearly Reporting, and Publication	

Subtask 1: Ongoing analysis of data generated during study progression	100% complete
Subtask 2a: Yearly Report (Year 1)	8/14
Subtask 2b: Yearly Report (Year 2)	8/15
Subtask 2c: Yearly Report (Year 3)	8/16
Subtask 2c: Final Report (Year 4)	10/17
Subtask 3: Publication of 2-3 peer-reviewed papers and 5-8 peer-reviewed conference proceedings disseminating investigations research	1 Paper Published; 2 in Prep. 10 Abstracts Published

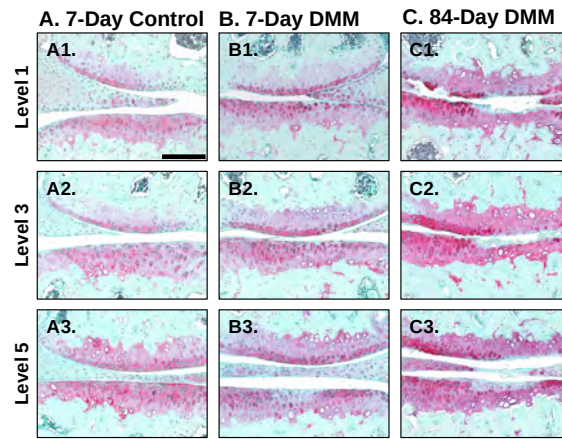
▪ **What was accomplished under these goals?**

Research Findings: Some Notable And Reported Highlights Of Our Research Findings From This Project Are As Follows:

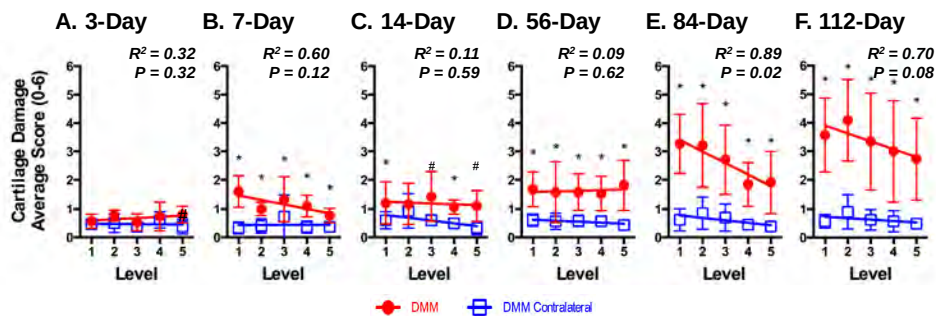
- **Early, Focal Changes in Cartilage Cellularity and Structure Following Surgically-Induced Meniscal Destabilization in the Mouse** – Published in David, et al. 2017 J Ortho Res. – Histological analysis of mouse joints following DMM demonstrated rapid (within 3-day of injury), and focal (spatially localized) changes in both the cellularity (loss of chondrocytes) and the structural integrity of articular cartilage. The findings shed new light onto the early pathology and time-course of PTOA development following DMM and suggest that acute changes to a specific and localized population of chondrocytes may be critical in the initiation of long-term cartilage degeneration following joint injury, and represent a potential target for chondo-protective/anti-osteoarthritic therapy. The findings are highlighted in the following figures and descriptions (**Fig 1 - 7**) findings published in David, et al. 2017 Journal of Orthopaedic Research.



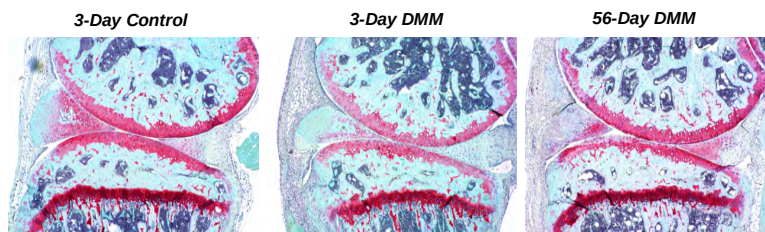
- Figure 1: Semi-quantitative histological scoring of the whole mouse joint demonstrated the rapid loss of proteoglycan and initiation of surface fibrillations [scores = .5-1] as early as 3-days in DMM joints, which stabilized as surface fibrillations and clefts between 7- and 56-days [scores = 1-2], before progressing into larger width- (C.) and depth-wise (D.) erosions [scores $\geq$ 3]. Similar trends were observed for the MFC (A. and B). These results illustrate the rapid nature of post-injury changes within mouse knees subjected to DMM surgery.



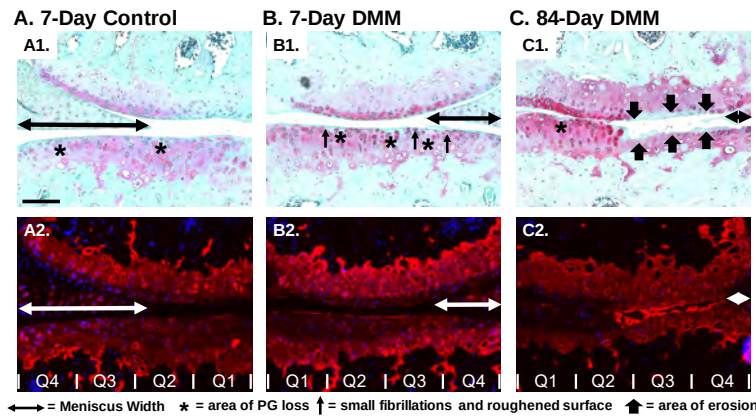
- Figure 2: Safranin-O staining of sections qualitatively highlighted the spatial and temporal changes that occur to the joint following DMM-injury. The medial meniscus extruded toward the medial margins of the joint resulting in the uncovering of 1/4th of the tibial plateau and femoral condyle articular cartilage. Changes in meniscal coverage appeared to coincide with both the distribution of cartilage damage from the anterior (level 1) to posterior (level 5) levels of the joint as well as medial-laterally within the joint, suggesting that the movement of the meniscus is critical factor in the initiation and location of cartilage damage following DMM.



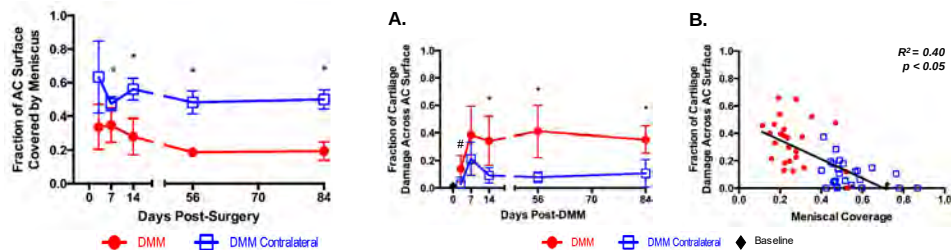
- Figure 3: Semi-quantitative scoring of cartilage damage in the medial femoral condyle confirmed the preferential anterior (level 1) vs. posterior (level 5) localization of cartilage damage that we observed visually following DMM-injury.



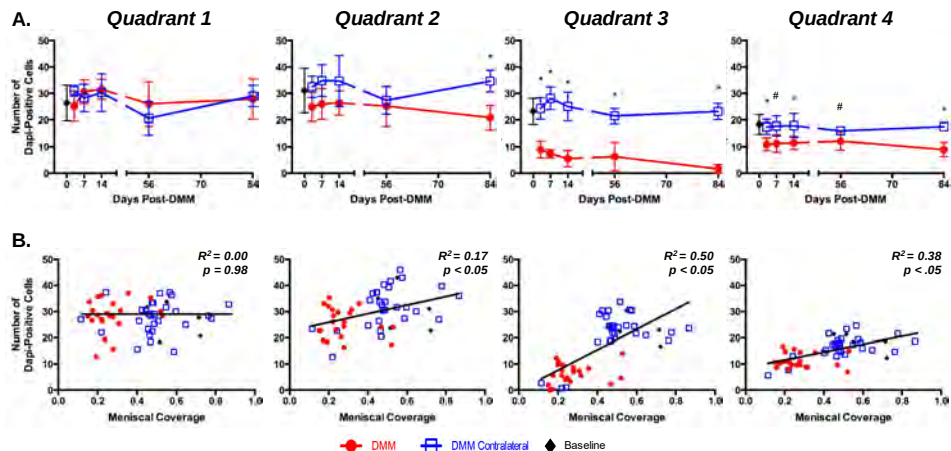
- Figure 4: A subset of DMM and contralateral joints that were sagittally sectioned and stained with Safranin-O/fast green to visual anterior-to-posterior progression of cartilage damage confirmed these findings.



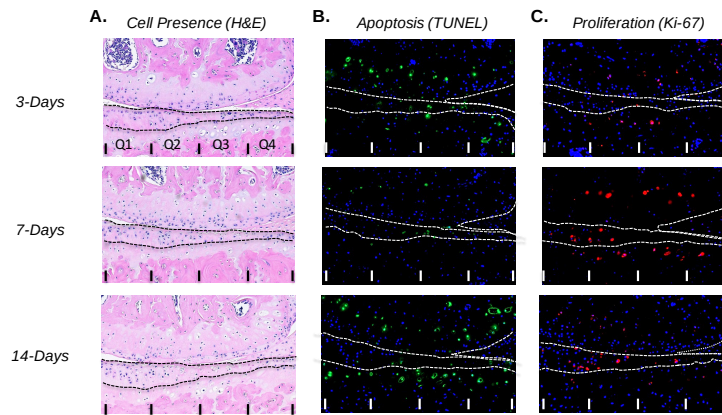
- Figure 5: Histological (Safranin-O/fast green [upper panel]) and immunohistochemical (type II collagen and DAPI [lower panel]) illustrated a distinct loss of both proteoglycan staining and chondrocytes, and increased type II collagen staining in regions of the MTP cartilage that col-localized with those experiencing a loss of meniscal coverage (specifically Quadrant 3 [Q3]). Again suggesting the importance of injury-related changes in the meniscus on cartilage health and the initiation of PTOA.



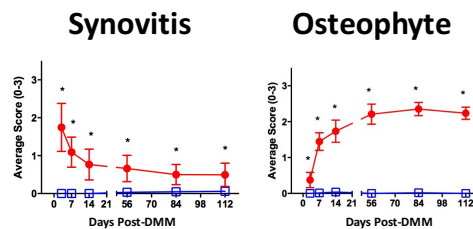
- Figure 6: Changes in the extent of meniscal coverage (extrusion) following DMM correlate with surface damage.



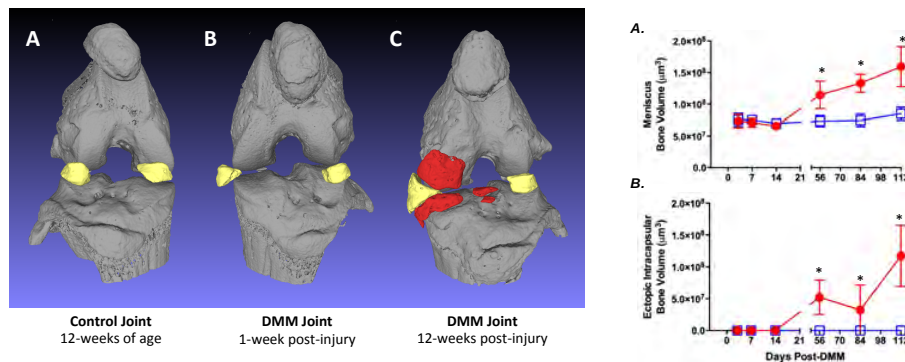
- Figure 7: The presence of chondrocytes across the medial tibial plateau following DMM, and their relationship with the extent of meniscal extrusion. Following DMM, extensive chondrocyte loss was observed within the articular cartilage quadrant experiencing meniscal uncovering (Q3), with loss only spreading to adjacent region (Q2 and 4) at later time points. The strong correlation between chondrocyte presence and meniscal coverage in quadrant 3 (and to a lesser degree Q2 & 4) highlights the importance of a stable joint in maintaining the cellular and structural health of the joint. These findings suggest that focal loss of chondrocyte at locations of meniscal uncovering may constitute the acute precipitating event that initiate PTOA in the DMM model. These cells appear to be a good target to leverage therapeutic strategies against.
- DMM Induces Rapid and Focal Progression of Chondrocyte Proliferation and Apoptosis** (David, et al. 2018 In Preparation): Following DMM-injury we observed the appearance of a distinct spatiotemporal progression of chondrocyte proliferation and apoptosis, which is suggestive of the focal activation of endochondral ossification like developmental process (strategies) with the articular cartilage following acute injury.



- Figure 8: Immediate (within 3-days) reductions in chondrocyte number was seen by H&E and DAPI in regions where the AC experienced acute altered femur-on-meniscus-on-tibia contact following injury (Q3). In these regions TUNEL staining co-localized with areas lacking DAPI-positive cells, consistent with these cells undergoing apoptosis. We also noted that from 3-days onward a spatiotemporal progression of proliferation and apoptosis was observed. Tissue regions initially exhibiting increased Ki67 expression (proliferation) at early timepoints subsequently exhibited increased TUNEL positive staining (apoptosis) and loss of chondrocytes, resulting in the lockstep spread of chondrocyte proliferation then apoptosis and cell loss to adjacent AC quadrants.
- Based upon these findings we suggest that chondrocyte proliferation may be a key, albeit detrimental, injury-response mechanism. We posit that altered loading patterns can activate chondrocyte proliferation and endochondral progression in chondrocytes. However, the production of matrix degrading enzymes and paracrine factors required to support these processes may further accelerate the degradation of an AC extracellular matrix that is already under aberrant mechanical loading, contributing to the vicious cycle of cartilage degeneration.
- Whole-Joint Structural Consequences Following Destabilization of the Medial Meniscus in the Mouse** (David, et al. 2018 In Preparation) – Histological and micro-CT analysis demonstrated the presence of significant whole joint structural changes within the knee joint following DMM surgery. Synovitis and osteophyte development occurred very rapidly and are key components of the early, natural pathology of the joint following injury. Enlargement and remodeling of the injured meniscus and ectopic calcification within the joint capsule occurred later in the disease progression, and have heretofore been overlooked in the study of this injury model.



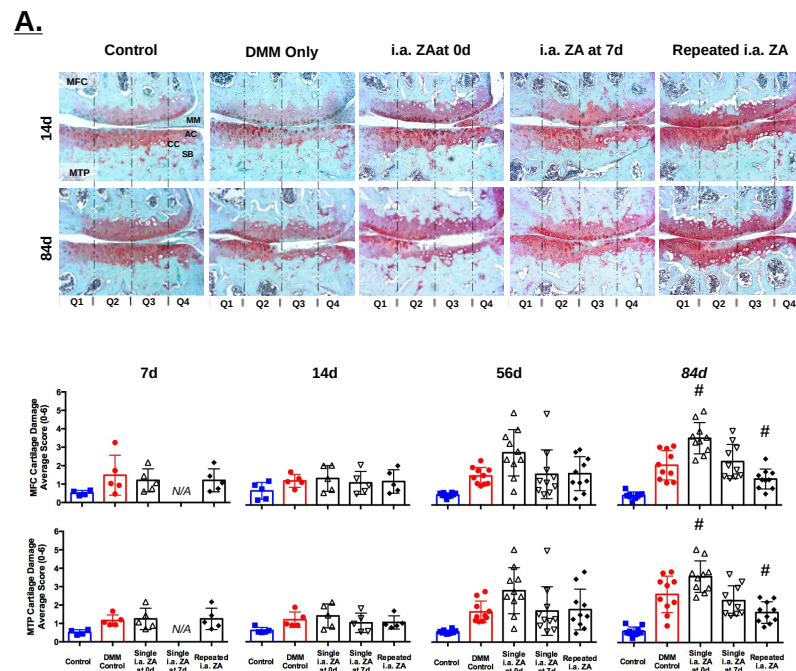
- Figure 9: In DMM-joints, but not SHAM or control joints, synovitis spikes rapidly (by 3-days post injury) then slowly subsides, but does not disappear, over the course of the study. Osteophyte development, through the formation of a cartilage template the subsequently calcification and bone formation, occurred very rapidly following DMM-injury.



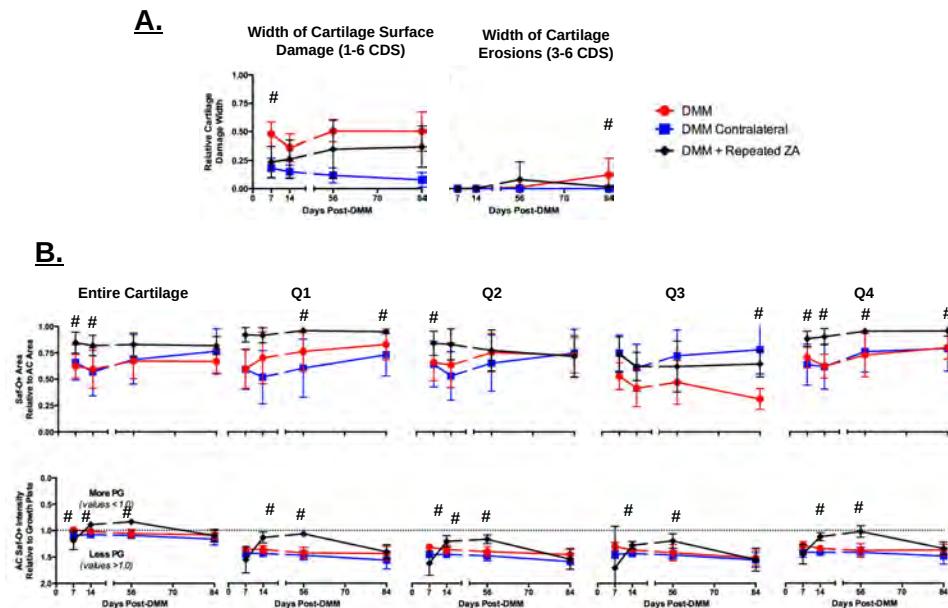
- Figure 10: Micro-CT analysis of the DMM joints demonstrate the presence of long-term, gross intra-capsular change within the joint. In healthy mice only the anterior and posterior menisci on the medial and lateral sides of the joint are calcified (yellow). Immediately following DMM, the menisci extrude medially from the joint contact, and at later

time points are observed to undergo significant increases in tissue and bone volume, and drastic changes in morphology. In DMM joints significant development of ectopic calcifications within the anterior-medial compartment of the joint were observed at 56-days post-DMM and beyond. These changes were not observed in SHAM and Control joints and have been previously unreported in the literature. We suggest that these observed increases in tissue calcification may represent a previous unrecognized response in the mouse knee to attempt to re-stabilize the joint following injury.

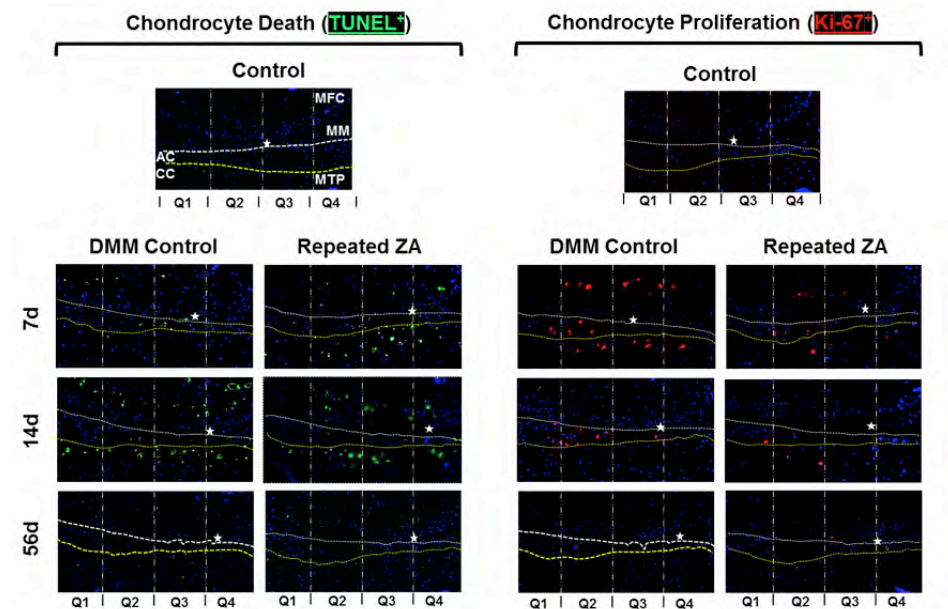
- **Repeated Intra-Articular Injection of Zoledronic Acid Suppresses Cartilage Erosions Following Destabilization of the Medial Meniscus in Mice** (David, et al. 2018 In Preparation) – The present study has shown that repeated i.a. injection of ZA into the murine knee following DMM provides cartilage protection by modulating the underlying processes of chondrocyte death, proliferation, and proteoglycan production in a spatiotemporal dependent manner. In addition to AC changes, ZA also appears to affect whole joint changes including changes in the menisci, ectopic bone, and osteophytes.



- Figure 11: Semi-quantitative histological scoring of articular cartilage damage revealed that repeated i.a. injection of ZA suppressed overt and 'deep' late-stage cartilage erosions in both the MFC and MTP of DMM joints; however, treatment provided minimal-to-no ability to modulate the earlier surface damage processes observed immediately following DMM (Figure 11A and B). Single i.a. injection administrations (either immediate [0d] or delayed [7d]) had markedly different effects on cartilage degeneration; joints administered one immediate i.a. dose of ZA (0d) experienced worsening of cartilage damage compared to DMM controls, while joints receiving one delayed i.a. dose of ZA (7d) exhibited damage no different than those of DMM controls (Figure 11A and B).

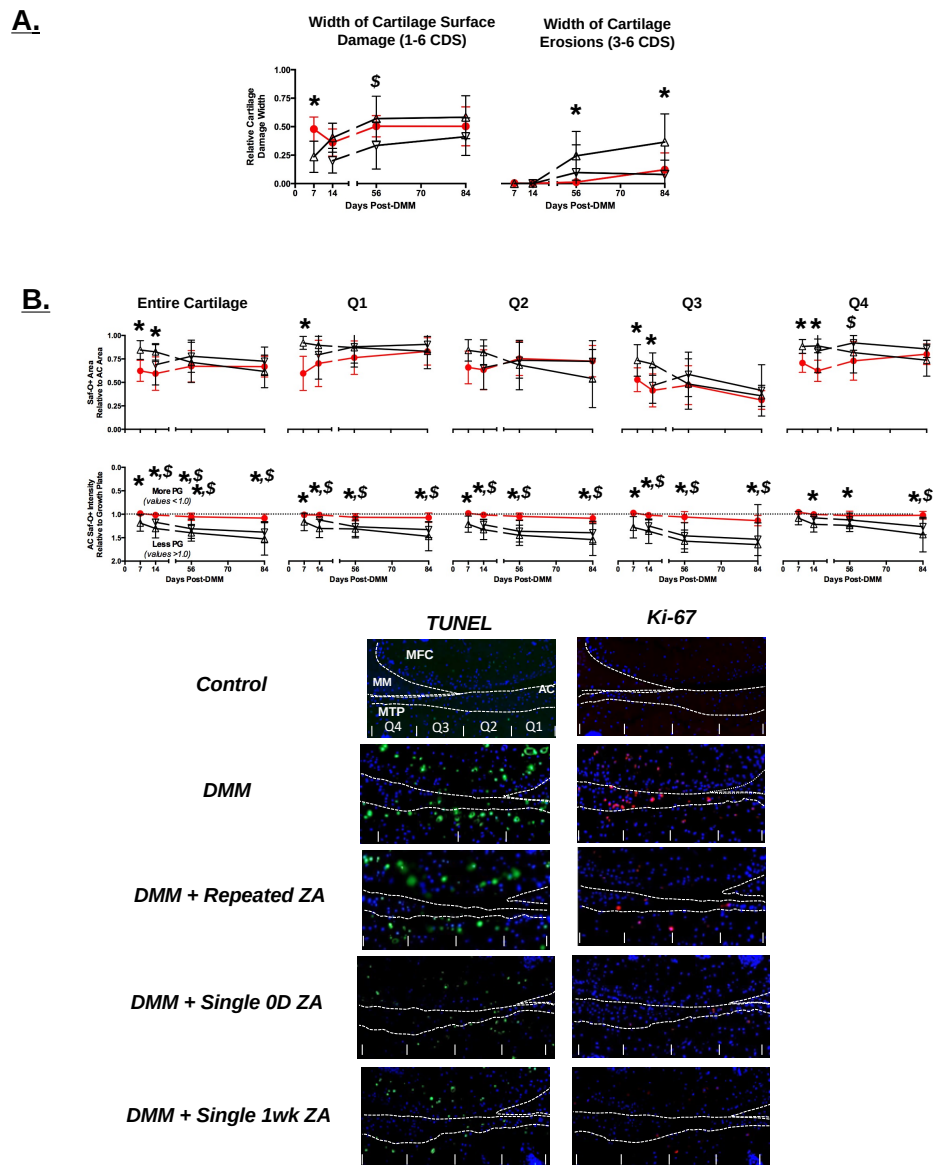


- Figure 12: Repeated i.a. injection of ZA A) mitigated both the width of surface tissue damage and the width of overt cartilage erosions (damage deeper than the superficial layer) observed across the medial tibial plateau of the injured mouse joint. Repeated local delivery of ZA promoted the retention, and even enhancement, of proteoglycan rich tissue (Safranin-O 'positive' area) and composition (Safranin-O Intensity) (B).

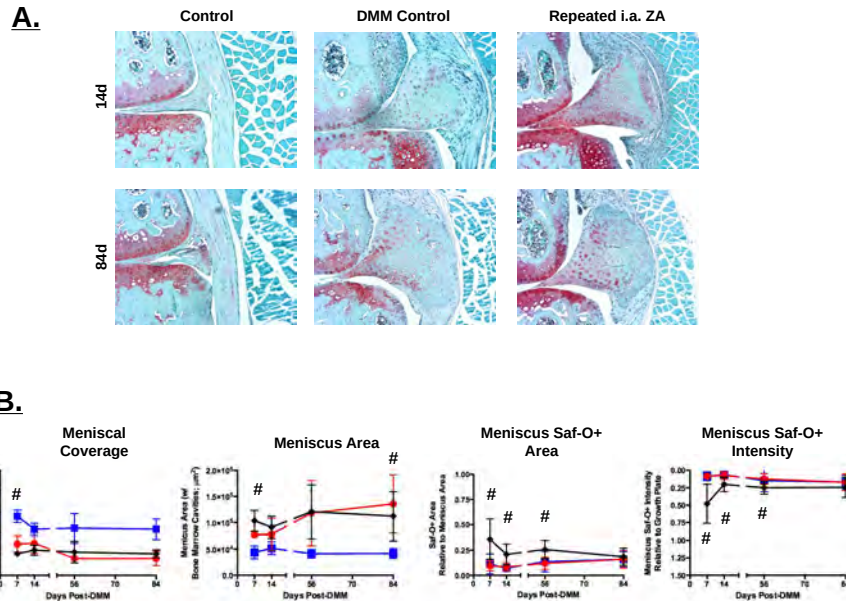


- Figure 13: The effect of DMM injury and i.a. ZA administration on cartilage cellular health, specifically chondrocyte death/apoptosis and proliferation, were assessed via immunohistochemistry using TUNEL staining and ki-67 antibody staining. With repeated i.a. ZA administration, there was a unique spatiotemporal effect on chondrocyte cell health. Interestingly, repeated i.a. ZA appeared to cause/accelerate chondrocyte loss/death within the medial tibial plateau quadrant (Q2) that experienced acute uncovering due to meniscal extrusion, while preventing chondrocyte loss in immediately adjacent quadrants, Q3 and Q4. In the CC, there was no appreciable long-term changes with regard to cell death and apoptosis. We also observed a transient increase Ki-67 staining in Q2, and minimally in Q1 and Q3, in the DMM control as early as 7d that diminishes by 56d (Figure 5); with repeated i.a. ZA, this proliferation was, qualitatively, almost completely suppressed by 14d. Similar findings are seen in both single i.a. groups in terms of proliferation and apoptosis compared to repeated i.a. ZA; however, there is no prevention of chondrocytes loss in Q3 and Q4 (Supplementary Figure 2A). These results suggest, interestingly, that ZA may aid in suppressing cartilage tissue damage by preventing the proliferation and accelerating the elimination of 'damaged' or activated chondrocytes in the region of cartilage that encounters injurious alterations in mechanical loading due

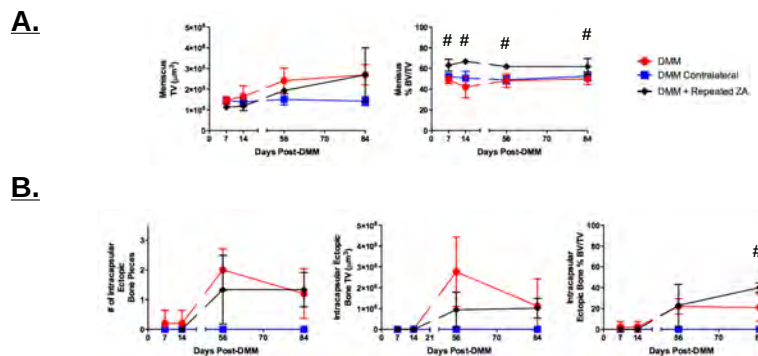
to injury-induced changes in meniscus-on-cartilage contact following DMM joint injury, while promoting the survival and metabolic efficiency of chondrocytes in the initially ‘undamaged’ regions adjacent to this quadrant. Our thesis is that by rapidly eliminating the activated/damaged cell populations from the compromised cartilage one may provide long-term protection against cell-mediated depredation of the cartilage matrix due to cell-mediated matrix degradation and remodeling.



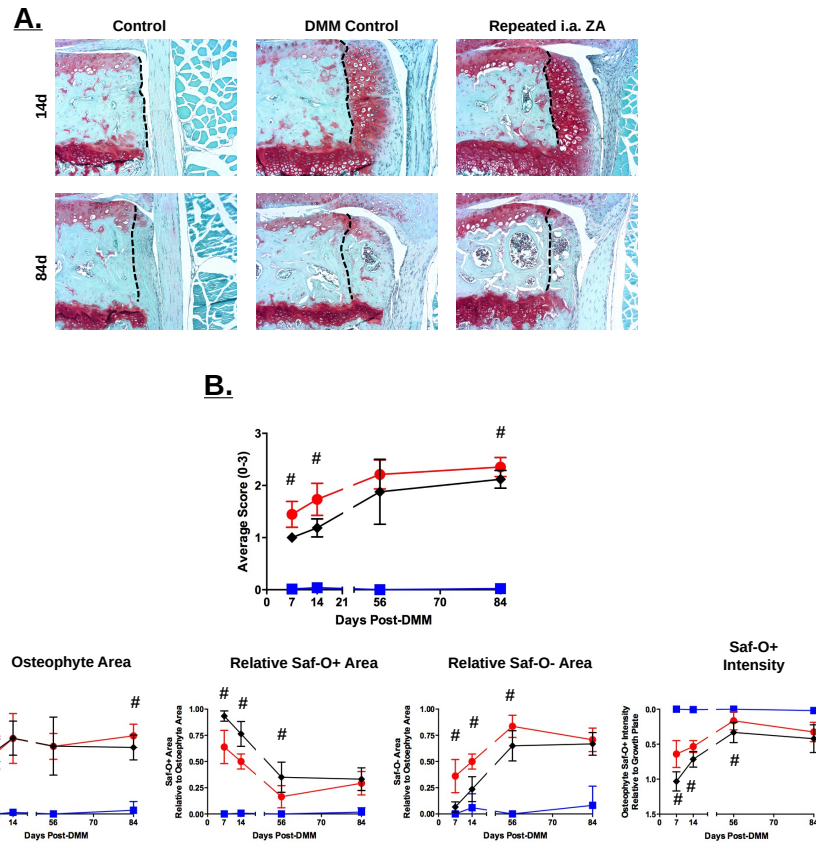
- Figure 14: We also established that single i.a. injections of ZA shortly following joint injury, either immediately at day zero (0d) or at one-week (7d) following injury were not as effective in modulating the control of cartilage damage and erosions, or in fully preventing protective cellular changes following DMM injury.
- Intra-Articular Injection Of Either Single Or Repeated Doses Of ZA Generated Differential Results With Regards To Their Influence On Synovitis, Osteophyte Maturation, And Ectopic, Intra-Capsular Calcification Following DMM.** (David, et al. 2018 In Preparation)



- Figure 15: As expected, repeated i.a. injection of ZA to the knee did not have the ability to modify the extrusion of the medial meniscus across the tibial plateau (A & B). Thus, the gross mechanical environment of both DMM and DMM + repeated i.a. ZA joint are expected to be largely the same. Additionally, using data collected via micro-CT we observed that even in the presence of ZA within the joint, the drug did not appreciably influence injury-related changes in the structure and composition of the meniscus following DMM.

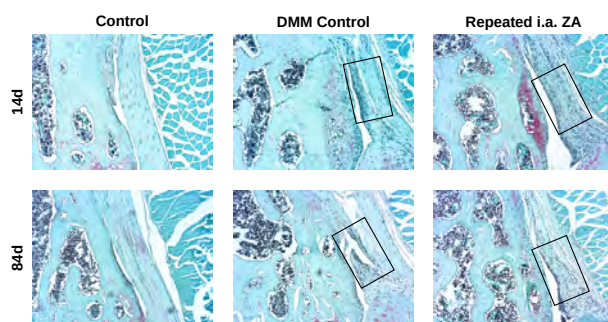


- Figure 16: However, as a result of the influence of bisphosphonates on the resorption and remodeling of calcified tissues, we showed that repeated i.a. ZA injections suppressed remodeling of the calcified cartilage templates and bone that form in the meniscus and ectopically in the joint capsule following injury. These results are fully expected given the mechanism of bisphosphonates once incorporated into newly forming calcified/bone tissues.

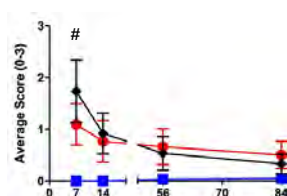


- Figure 19: While i.a. ZA administration does not prevent the ultimate formation of osteophytes in injury destabilized mouse knees, repeated administration does delay the rapidity of osteophyte formation when assessed visually (A), semi-qualitatively (B), and qualitatively (C).

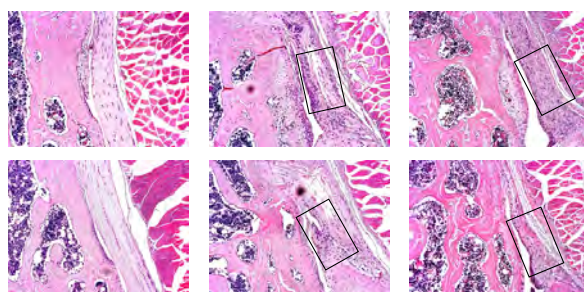
A.



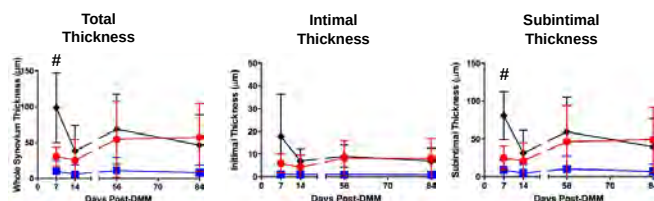
B.



C.



D.



- Figure 20: Lastly, despite findings that bisphosphonates such as ZA have anti-inflammatory properties in joint diseases such as rheumatoid arthritis, as well as tumor/cancer suppression, we did not observe any significant influence of repeated i.a. ZA administration on synovitis and joint inflammation as assessed visually (A & C), semi-quantitatively (B) and quantitatively (D).

What opportunities for training and professional development has the project provided?

The Following Students, Staff, And Faculty Were Provided Training Opportunities Through This Award During The Last Reporting Period

Graduate Student Training:

- Michael David – University of Delaware Biomedical Engineering Graduate Student (4th-year Ph.D. Student; student lead on project; supported by award)
- Brian Graham – University of Delaware Mechanical Engineering Graduate Student (4th-year Ph.D. Student; assistance with data analysis)
- Ryan McDonough - University of Delaware Biomedical Engineering Graduate Student (2nd-year Ph.D. Student; assistance with experiments)

Undergraduate Student Training:

- *Brianna Hulbert - University of Delaware Biomedical Engineering Undergrad (Sophomore; Summer Scholar researcher and Independent Study Researcher 2016-2017)*
- *Sejal Shah - University of Delaware Biomedical Engineering Undergrad (Sophomore; Summer Scholar researcher and Independent Study Researcher 2016-2017)*
- *Alexis Merritt - University of Delaware Biomedical Engineering Undergrad (Sophomore; Summer Scholar researcher and Independent Study Researcher 2016-2017)*
- *High School Student Training:*
 - *Pooja Kaji - Wilmington Charter School, DE - Rising Senior (UD Summer 2015 K12 Program)*
- *Staff Training:*
 - *Melanie Smith – Research Technician University of Delaware Biomedical Engineering (staff lead on project; partially supported by award, resigned from position in April, 2017)*
- *Faculty Training:*
 - *Christopher Price – Assistant Research Professor, University of Delaware Biomedical Engineering (PI on project) – During the reporting period Dr. Price attended the University of Pennsylvania's Cartilage Repair Symposium in Philadelphia, PA, the 2017 Summer Biomechanics, Bioengineering, and Biotransport Conference (SB3C) in Tucson, AZ, the 2017 Biomedical Engineering Society (BMES) meeting in Phoenix, AZ, and the 2016 Musculoskeletal Biology and Engineering Gordon Research Conference to increase his knowledge in the field of cartilage biology and repair, and orthopaedic biomechanics, respectively. During the prior reporting periods Dr. Price attend multiple UPenn Cartilage Repair Symposia, BMES, SB3C, and Orthopedics Research Society (ORS) conferences for the same purposes.*

The Following Additional Students Were Provided Training Opportunities Through This Award During The Previous Reporting Periods

- *Graduate Student Training:*
 - *Janty Shoga – University of Delaware Biomechanics and Movement Science Graduate Student (M.Sc. Student Graduated 7/2016; assistance with data analysis)*
- *Undergraduate Student Training:*
 - *Avery White - University of Delaware Biomedical Engineering Undergrad (Graduated 5/2016)*
 - *Ryan Locke - University of Delaware Biomedical Engineering Undergrad (Graduated 5/2016)*
 - *Brendan Horne - University of Delaware Biomedical Engineering Undergrad (Graduated 5/2016)*
 - *Brianna Geitter - University of Delaware Biomedical Engineering Undergrad (Graduated 5/2016)*
 - *John Lowman - University of Delaware Biomedical Engineering Undergrad (Graduated 5/2017)*
 - *Rachel Pilachowski - University of Delaware Biomedical Engineering Undergrad (Graduated 5/2017)*
 - *Srinivasa Gajjala - University of Delaware Biomedical Engineering Undergrad (Graduated 12/2017)*
 - *Christopher Hernandez - University of Delaware Biomedical Engineering Undergrad (Expected Graduation 2018)*
 - *Malhar Sakarwala - University of Delaware Biomedical Engineering Undergrad (Expected Graduation 2018)*
- *High School Student Training:*
 - *Fiona Flowerhill - Wilmington Charter School, DE - Rising Senior (UD Summer 2015 K12 Program)*
 - *Oliva Laxton – Strath Haven High School, PA – Rising Senior (UD Summer 2015 K12 Program)*
 - *Shu-Jin Kust – Charter School of Newark DE - Rising Junior (UD Summer 2016 K12 Program)*
 - *Michael Lan – Wilmington Charter School, DE - Rising Senior (UD Summer 2016 K12 Program)*
- **How were the results disseminated to communities of interest?**

Results of the research were disseminated to the general public in following ways:

 - *First, through the incorporation of research methodologies and research findings in the Biomedical Engineering core curriculum course that the PI (Price) teaches at the University of Delaware. Examples of the socioeconomic concerns of PTOA and the present findings of the PI's research in PTOA funded by this award were incorporate into the course lecture on the pathophysiology of the musculoskeletal system.*

- Second, research images produced through the present project were displayed at the Annual Science in Art Symposia at the University of Delaware in 2016 and 2017. The symposia were sponsored by the UD BME Department and highlighted the research work being performed at the University through the display and discussion of artistic images produced through research. Three students present over a dozen pictures/artwork that were derived from the present work.
- Third, students engaging in the present work utilized three separate media outlets to advertise and describe the work that they were performing in conjunction with the present award.
 - In 2014 Michael David was invited to be a guest on the student run University of Delaware Radio 91.3 WVUD Science Rocks show and podcast (<https://sites.google.com/site/sciencerocksonline/nhs>), which aired locally in the Delaware area, to describe his research on this project.
 - Michael David and Melanie Smith also were invited onto the student run WVUD show and podcast Rise and Science in 2016 (<http://www.udriseandscience.com/podcasts/2016/8/29/episode-12-michael-david-melanie-smith-post-traumatic-osteoarthritis>) to discuss our work on this project as well.
 - Additionally, Avery White, while an undergrad in the lab, was interviewed for the website LabTV in 2014 (<http://www.labtv.com/Home/Profile?Area=&researcherId=1178>). LabTV spotlight researchers from many of the world's leading universities, government labs, and corporations – people and places on the forefront of a growing revolution in healthcare and technology. Avery's interview regarding our research can also be found on YouTube (<https://www.youtube.com/watch?v=QsG-Rr3vtw0>). Possibly of ever greater significance, Avery's interview was subsequently highlighted on Dr. Francis Collins' NIH Director Blog in 2015 (<https://directorsblog.nih.gov/2015/06/25/labtv-curious-about-post-traumatic-osteoarthritis/>).
- Additionally, as per convention in fields of biomedicine and biomedical engineering, the results and findings of the present work were disseminated to the research community through .
- **What do you plan to do during the next reporting period to accomplish the goals?**
 - "Nothing to Report."

• **IMPACT:**

- **What was the impact on the development of the principal discipline(s) of the project?**
 - Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).
 - As a result of the findings of this research, we and the field of PTOA biology now have an increased appreciation for the early and focal changes in cellular health that accompany the destabilization of the joint following acute injury. We have demonstrated the critical role that the configuration of the meniscus plays in the initiation and progression of chondrocyte death and cartilage structural changes following injury.
 - We have also demonstrated within the present injury-induced PTOA model, the rapidity and focal nature of chondrocyte death following injury. These findings present researchers a distinct population of cells against which the study of disease progression at the molecular and cellular level can now be directed. In addition, the behavior of this population of cells indicates that these specific cells may be the focal initiators of PTOA development and progression and may constitute a population of cells (and pathology) that can be targeted via chondro-protective (or anti-osteoarthritic) therapies to try to delay or prevent their death and subsequent tissue degeneration.
 - These findings, identified in a small animal model of PTOA, have distinct similarities/correlates to processes observed in larger models as well as clinically in humans. Therefore, the specific processes and results identified herein can guide the investigation of similar PTOA pathologies within larger pre-clinical injury models, as well as in the clinical diagnosis and understanding of PTOA following acute knee injury.
 - We have also demonstrated that intra-articular injections of zoledronic acid, if administered repeatedly (weekly) following joint injury in the present model can provide a degree of anti-osteoarthritic efficacy. Our data also suggest that repeated i.a. injection of ZA can modulate cartilage degeneration by accelerating the removal of injured/activated chondrocytes from regions of cartilage that experience both acute and chronic changes in loading and articulation due to injury, while promoting the health, survival, and anti-osteoarthritic fate/metabolism of chondrocytes adjacent to the regions that appear to be activated by changes in loading and contact following injury. We posit that by eliminating activated/injured/dysfunctional chondrocytes from regions of acutely 'damaged'

cartilage one may provide long-term protection against cell-mediated degradation of the cartilage matrix due to cell-mediated matrix degradation and remodeling.

- *These findings support and corroborate ex vivo and in vitro findings from our collaborator (X Lucas Lu, Project-PI), and highlight the potential for i.a. ZA as a therapeutic approach for mitigating the long-term effects of knee-injury on PTOA. These results support the further pre-clinical investigation of the present findings and the need for continued translational studies to refine the use of i.a. ZA as a PTOA therapeutic.*
- **What was the impact on other disciplines?**
 - *While not yet directly applicable to the treatment of cartilage damage clinically following joint injury, these findings highlight the need to consider the immediate/acute changes occurring to the joint following injury in both the clinical study and care of patients. These results suggest that due consideration to the needs of joint stability following injury should be considered by clinical practitioners in the treatment and care of injured patients/service member. These findings also suggest that further research into the immediate post-injury changes within the knee should be a clinical priority, thus defining the timeline and specific pathophysiology of the disease in the clinical population. We suspect that a similar etiology should present clinically and would be a critical piece of knowledge in directing the treatment of joint injury in the attempt to prevent long-term PTOA.*
- **What was the impact on technology transfer?**
 - *Nothing to Report*
- **What was the impact on society beyond science and technology?**
 - *Ultimately, we hope that this basic science and translational research can lead to breakthroughs in the development of clinical treatments strategies, possibly based on local targeted (i.e. intra-articular) administration of bisphosphonates, that can be applied to patients in the general population in the early, acute phase following joint injuries in order to prevent or delay the onset of cartilage damage, degeneration, and disease, and thus begin to relieve the significant socioeconomic burden of PTOA to both the military, the taxpayers, and the general population.*
- **CHANGES/PROBLEMS:** *The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*
 - **Changes in approach and reasons for change**
 - *Nothing to Report*
 - **Actual or anticipated problems or delays and actions or plans to resolve them**
 - *Nothing to Report*
 - **Changes that had a significant impact on expenditures**
 - *Nothing to Report*
 - **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
 - *Nothing to Report*
- **PRODUCTS:**
 - **Publications, conference papers, and presentations**
 - **Journal publications.**
 - *David MA, Smith MK, Pilachowski RN, White AT, Locke RC, Price C#. Early, focal changes in cartilage cellularity and structure following surgically induced meniscal destabilization in the mouse. J Orthop Res. 35(3) 2017:537-547. Published - doi: 10.1002/jor.23443. PubMed PMID: 27664978. Federal funding support acknowledged.*
 - **Other publications, conference papers, and presentations.**
 - *Manuscripts in Preparation*
 - *David, M.A., Smith, M.K., Merit A., Shah, S., Pilachowski, R., Hulbert, B., Locke, R., Lu, X.L., Price, C. Repeated Intra-Articular Injection of Zoledronic Acid Prevents Cartilage Erosions by Modulating Chondrocyte Death, Proliferation, and Proteoglycan Production in a Murine Model of Post-Traumatic Osteoarthritis (In Preparation; Expected 2018)*
 - *David, M.A., Smith, M.K., Graham, B.T., Lowman, J., Lu, X.L., Price, C. Whole Joint Changes Associated with Repeated Intra-Articular Injection of Zoledronic Acid After Destabilization of the Medial Meniscus in Mice. (In Preparation; Expected 2018)*

Conference Abstracts/Proceedings Since in Last Reporting Period

- David, M.A., Smith, M.K., Graham, B.T., Merritt, A., Shah, S., Pilachowski, R.N., Hulbert, B., Price, C. Repeated intra-articular injection of zoledronic acid modulates chondrocyte proliferation and death in murine post-traumatic osteoarthritis. Orthopaedic Research Society (ORS) Annual Meeting. New Orleans, LA. March 2018 (Podium Presentation)
- David, M.A., Smith, M.K., Pilachowski, R.N., Lowman, J., Price, C. Repeated intra-articular injection of zoledronic acid suppresses cartilage erosions after DMM. Biomedical Engineering Society (BMES) Annual Meeting. Phoenix, AZ. October 2017 (Podium Presentation)
- David, M.A., Smith, M.K., Merritt, A., Pilachowski, R.N., Lan, M., Shah, S., Locke, R.C., Price, C. Destabilization of the Medial Meniscus in Mice Induces Rapid and Focal Progression of Chondrocyte Proliferation and Death. Orthopaedic Research Society Annual Meeting, San Diego, CA, March 2017 (Poster Presentation)
- David, M.A., Smith, M.K., Pilachowski, R.N., White, A.T., Locke, R.C., Price, C. Destabilization of the Medial Meniscus in Mice Induces Early, Focal Changes in Cartilage Cellularity and Structure. Orthopaedic Research Society Annual Meeting, San Diego, CA, March 2017 (Poster Presentation)
- David, M.A., Smith, M.K., Pilachowski, R.N., White, A.T., Locke, R.C., Lowman, J.M., Gietter, B., Horne, B., Graham, B.T., Price, C. Whole-Joint Structural Consequences Following Destabilization of the Medial Meniscus in the Mouse. Orthopaedic Research Society Annual Meeting, San Diego, CA, March 2017 (Poster Presentation)
- David, M.A., Smith, M.K., Pilachowski, R.N., Lowman, J.M., Gietter, B., Price, C. Repeated Intra-Articular Injection of Zoledronic Acid Suppresses Cartilage Erosions Following Destabilization of the Medial Meniscus in Mice. Orthopaedic Research Society Annual Meeting, San Diego, CA, March 2017 (Poster Presentation)
- David, M.A., White, A.T., Pilachowski, R.N., Locke, R.C., Smith, M.K., Price, C. (2016) Spatio-Temporal Quantification of Cartilage Structural Changes in a Murine Model of Post-Traumatic Osteoarthritis, Summer Biomechanics, Bioengineering and Biotransport Conference (SB3C). National Harbor, MD. June 2016 (Podium Presentation)

Conference Abstracts/Proceedings During the Previous Reporting Periods

- David, M., Smith, M., White, A., Locke, R., Price, C. (2015) Quantification of Early Structural Joint Changes in a Murine Model of Post-Traumatic Osteoarthritis. Biomedical Engineering Research Society Annual Meeting. Tampa, FL. October 2015 (Poster Presentation)
- White, A., David, M., Smith, M., Pilachowski, R., Price, C. (2015) Post-traumatic Osteoarthritis Dual Quantification System of Cartilage Degradation in Murine Model Following Joint Destabilizing Surgery. Biomedical Engineering Research Society Annual Meeting. Tampa, FL. October 2015 (Poster Presentation)
- David, M.A., Smith, M.K., White, A.T., Locke, R.C., Price, C. (2015) Detailed Quantification of Early Articular Cartilage Structural Changes in the Murine Destabilized Medial Meniscus Model of Post-Traumatic Osteoarthritis. 60th Annual Meeting of the Orthopaedic Research Society, Las Vegas, NV. (Poster Presentation)

Invited Presentations

- **Price, C.** (2015) Targeted Prevention Strategies for Post-Traumatic Osteoarthritis, 2015 Penn Cartilage Repair Symposium, University of Pennsylvania, Philadelphia, PA

▪ **Website(s) or other Internet site(s)**

- <http://sites.udel.edu/pricegroup/> - this site is the homepage of the Price Musculoskeletal Mechanotransduction and Mechanobiology Laboratory at the university of Delaware.

▪ **Technologies or techniques**

Nothing to Report

▪ **Inventions, patent applications, and/or licenses**

Nothing to Report

▪ **Other Products**

Nothing to Report

• **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

▪ **What individuals have worked on the project?**

- *Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged*

from a previous submission, provide the name only and indicate "no change."

Example:

Name:	<i>Christopher Price</i>
Project Role:	<i>Partnering PI</i>
Nearest person month worked:	<i>2</i>
Contribution to Project:	<i>Dr. Price oversaw the design, management, conduct, analysis, and dissemination of the present work. He served as the primary PI in charge of the in vivo animal injury and treatment studies conducted in the present work. He retained primary oversight over all of the staff and students employed in the present work.</i>
Funding Support:	<i>Dr. Price was supported by the University of Delaware, the NIH, and the DoD during the conduct of the present study.</i>

Name:	<i>Michael A. David</i>
Project Role:	<i>Graduate Student</i>
Nearest person month worked:	<i>12</i>
Contribution to Project:	<i>Mr. David was the primary graduate student supported by the present work. He oversaw the day-to-day conduct of the research following the resignation of Ms. Melanie Smith (Research Technician). Michael's role in the study was the performance of all experimental procedures and studies associated with tissue histology and immunohistology during the study, as well as all data analysis and writing. Mr. David also assisted with the animal husbandry, surgeries, and treatment aspects of the study. Mr. David was also primarily responsible for the training and immediate oversight of the 12 undergraduate and 5 high school students that contributed to the conduct and completion of the present work.</i>
Funding Support:	<i>Mr. David was supported by the University of Delaware, the NIH, and the DoD during the conduct of the present study.</i>

Name:	<i>Melanie K. Smith</i>
Project Role:	<i>Research Technician</i>
Nearest person month worked:	<i>6</i>
Contribution to Project:	<i>Mrs. Smith served as the primary Research Technician for the present project. Her role was to oversee the day-to-day conduct of the research and for the day-to-day management of the students involved on the project. Melanie also was primarily responsible for all of the animal husbandry, surgeries, and treatment aspects of the study. Mrs. Smith also assisted with the scoring and analysis of histological specimens obtained throughout the study.</i>
Funding Support:	<i>Ms. Smith was supported by the University of Delaware, the NIH, and the DoD during the conduct of the present study.</i>

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
 - *In April of 2017, Ms. Smith (Research Technician) resigned her position in the lab in order to take another job position at the University of Pennsylvania. As the grant was in its No Cost Extension period and all funds for Ms. Smith's salary had been previously utilized and she was being paid through laboratory start-up funds, no change in budgeting was required. Ms. Smith's position was not filled following her resignation since all live animal work had been completed.*

- **What other organizations were involved as partners?**
 - *Nothing to Report*
- **SPECIAL REPORTING REQUIREMENTS**
 - **COLLABORATIVE AWARDS:** *Nothing to Report*
 - **QUAD CHARTS:** *Nothing to Report*
- **APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc. Reminder: Pages shall be consecutively numbered throughout the report.*

Early, Focal Changes in Cartilage Cellularity and Structure Following Surgically Induced Meniscal Destabilization in the Mouse

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ABSTRACT: Post-traumatic osteoarthritis (PTOA) is an accelerated form of osteoarthritic cartilage degeneration affecting approximately 20–50% of patients experiencing joint injury. Currently PTOA is incurable; to better understand the etiology of PTOA and to develop rational anti-osteoarthritic therapies, it is critical to understand the spatiotemporal initiation and the progression of PTOA. In this study, we employed semi-quantitative histological scoring and quantitative damage analysis to examine disease progression in the murine destabilization of the medial meniscus (DMM) model of PTOA from early (3 days) through late- (112 days) disease timepoints. We observed significant, progressive articular cartilage (AC) cellular, and structural changes in the medial compartments of injured joints as early as 3 days. Spatially within the joint, cartilage damage (erosions) were observed anteriorly at 84 days. Furthermore, a drastic loss in chondrocyte number (by 3 days), surface damage (at 7 days), and cartilage erosion (at 84 days) was found to co-localize to the specific region of the medial tibial plateau AC that experienced a change in meniscal coverage due to meniscal extrusion following DMM. Taken together, these results suggest that DMM-mediated extrusion of the medial meniscus leads to rapid, spatially dependent changes in AC cellularity and structure, and precipitates the focal degeneration of cartilage associated with PTOA. Importantly, this study suggests that joint instability injuries may trigger immediate (<3 days) processes within a small population of chondrocytes that directs the initiation and progression of PTOA, and that development of chondroprotective strategies for preventing and/or delaying PTOA-related cartilage degeneration are best targeted toward these immediately early processes following joint injury. © 2016 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. *J Orthop Res*

Keywords: post-traumatic osteoarthritis; spatiotemporal cartilage damage; chondrocyte loss; meniscal extrusion; destabilization of the medial meniscus (DMM)

Approximately 20–50% of patients suffering traumatic joint injury, e.g., anterior cruciate ligament tears, articular fracture, meniscal tears, or meniscectomies, will develop post-traumatic osteoarthritis (PTOA) within 10–15 years of injury.^{1,2} Currently, PTOA is not preventable and the precise mechanisms by which acute joint injury leads to long-term cartilage degeneration remains unknown² partly due to: (i) limited availability of early disease stage human cartilage; (ii) limited in vivo cartilage assessment techniques; and (iii) the long timespan of clinical disease progression. To overcome these limitations and to define the mechanisms underpinning PTOA initiation and progression, cartilage degeneration has been studied in numerous pre-clinical small-animal models of joint injury.

The destabilization of the medial meniscus (DMM), a surgically induced joint instability model, is a commonly used murine model of PTOA due to its ease of implementation, high reproducibility, and pattern of disease progression thought to closely recapitulate human osteoarthritis.³ Collectively, murine DMM studies have identified mild cartilage damage in early disease (7- and 14 days)^{4–9} and mild-to-severe cartilage loss/erosion at mid-to-late stage disease (28 through 112 days).^{4–25} Typically, cartilage damage severity and progression has been

evaluated using whole-joint level semi-quantitative histological scoring systems.^{20,26–28} However, such systems have limited ability to fully characterize early changes in articular cartilage (AC) degeneration because they rely on: (i) binary metrics of superficial tissue damage (fibrillations and clefts), which only indicate the presence of these changes not their spatial extent; and (ii) metrics of cartilage erosion that simultaneously incorporate width- (across the surface) and depth-wise (into the tissue) damage components in their analysis. Furthermore, scores are oftentimes presented as a single aggregated damage metric derived from the maximal extent of damage within a section or across a joint compartment, or summed/averaged across the joint. Thus, while semi-quantitative scoring systems have helped to define the time-course of whole-joint level cartilage damage post-DMM, they can inadvertently mask key aspects of the spatial initiation and progression of cartilage degradation.

Defining the anterior-to-posterior and medial-to-lateral distribution of cartilage damage may provide critical insight into the mechanisms by which PTOA initiates and progresses. In the pioneering DMM study by Glasson et al.,³ qualitative observations of cartilage erosions being preferentially localized to anterior-to-central regions of the medial tibial plateau (MTP) of DMM joints were made. Furthermore, while not addressed in prior studies, our qualitative impressions of the murine DMM literature suggests a co-localization of cartilage damage to medial regions of the MTP that were previously covered by the medial meniscus (MM).^{7,9,14–16,20–24} Supporting these impressions, studies utilizing DMM

Conflicts of interest: None.

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within various species have indicated increased joint laxity and anterior translation (mice),²⁹ altered medial compartment contact stresses (mice and rabbit),^{10,30} and cartilage damage to the middle and outer MTP (rat).³¹ Furthermore, non-invasive murine models of PTOA support the co-localization of applied load, cartilage damage, and chondrocyte apoptosis.³² While a few murine studies have identified increased chondrocyte death post-DMM,^{4,6,24,27,33} the details regarding the spatial distribution of chondrocyte loss across the AC and their relationship to meniscal extrusion have yet to be investigated.

Taken together, we believe that meniscal extrusion post-DMM plays an important role in the rapid, focal initiation, and progression of cartilage damage. We hypothesized that chondrocyte loss and cartilage structural damage would rapidly (≤ 7 days) and preferentially localize to cartilage regions where DMM-induced MM extrusion results in altered femur-on-meniscus-on-tibia contact. However, the use of conventional semi-quantitative scoring alone would likely be unable to address such relationships. Therefore, we utilized a set of spatiotemporal scoring and evaluation methods to address this hypothesis, and to elucidate the relationship between meniscal extrusion and changes in cartilage cellularity and structure from early (3-day) through late-stage (112 days) disease in the murine DMM model of PTOA.

METHODS

Animals and Surgeries

Male C57BL/6 mice ($n=83$) were purchased from the Jackson Laboratory at 9 weeks of age and housed in standard cages (≤ 5 mice/cage). Mice were maintained on a 12-h light–dark cycle in a climate controlled vivarium with access to food (Prolab RMH 3000) and water ad libitum. Mice were randomly assigned to one of three groups: DMM, sham, or baseline/age-matched controls.

At 12 weeks of age, right knees of mice in the DMM group ($n=5$ –10 mice/group) underwent the DMM surgery^{3,21}: contralateral limbs served as un-operated internal controls. Briefly, mice were anesthetized using inhaled isoflurane and prophylactically administered antibiotic and analgesic. Using aseptic techniques, the medial meniscotibial ligament (MMTL) was exposed and transected to release the anterior horn of the MM from the tibia and surgical incisions were subsequently closed. A subset of mice underwent sham surgery, identical to the DMM surgery, except transection of the MMTL was omitted. Following surgery, mice recovered quickly to full ambulation and unrestricted cage activity. Collection of tibiofemoral joints occurred upon euthanasia (CO_2 exposure and cervical dislocation) at either 3-, 7-, 14-, 56-, 84-, or 112 days post-injury, or 0 days for non-surgical baseline controls. All animal procedures were approved by the University of Delaware's Institutional Animal Care and Use Committee (Protocol Number 1252).

Histological Processing and Staining

Following tissue collection, joints were processed for paraffin embedding, sectioning, and histological staining using standard techniques.²⁸ Briefly, all joints were placed within

tissue cassettes at a flexion angle of $\sim 80^\circ$, approximating the joint's natural flexed position. Specimens were then fixed in 4% paraformaldehyde for 48-h at 4°C , decalcified in 14% EDTA (pH 7.4) over 15 days at 4°C , and processed for paraffin embedding. Joints were serially cut into 5- μm thick coronal sections and placed on charged slides (two sections/slide). Starting at the front of the joint (anterior), every 10th slide (every ~ 100 - μm) was stained with 1% Safranin-O, 0.02% fast green, and Weigert's iron hematoxylin. From these, a subset of five slides spanning ~ 500 - μm of the joint and centered about the tibial plateau and femoral condyle cartilage contact region were selected for damage analysis (Fig. 1A). These slides were labeled (1–5), from the anterior to the posterior level of the joint.

Spatiotemporal Scoring of Width- and Depth-Wise Cartilage Damage

Semi-quantitative scoring of cartilage damage was performed across all stages of disease development (3- to 112 days). Three blinded scorers (MAD, MKS, and ATW or RNP) scored the degree of cartilage damage present in each slide using two separate semi-quantitative scoring metrics to evaluate the extent of width- versus depth-wise cartilage damage. Width-wise damage across the articular surface was assessed (Fig. 1C, Table 1) using a modified version of the OARSI scoring system.²⁸ Depth-wise cartilage damage, relative to the calcified cartilage (CC)/subchondral bone interface (Fig. 1C, Table 1), was assessed using an adapted version of Chambers' et al.²⁶ scoring system. For each metric, cartilage damage was assessed on a 0–6 scale. Scores of 0–2 were applied identically within both scoring systems, while scores of 3–6 differed. This allowed for classification of cartilage erosion based on its width-wise extent independent of depth-wise extent, and vice versa. Scoring was performed and aggregated separately for each of the four compartments of the joint: the MTP, the medial femoral condyle (MFC), the lateral tibial plateau (LTP), and the lateral femoral condyle (LFC) (Fig. 1B). At each level, scores (anterior [1] to posterior [5]) were aggregated across observers within an individual joint. For overall temporal analysis, scores from each level were averaged to generate a whole-joint damage score used to calculate group means. For spatial analysis of anterior-to-posterior damage, the average individual specimen score at each level was used to calculate a group mean for each level and timepoint.

Quantification of Meniscal Coverage of the Cartilage Surface, Cartilage Damage, and Chondrocyte Cellularity

To establish spatial (medial-to-lateral) relationships between meniscal extrusion and changes in chondrocyte cellularity and cartilage damage within the medial joint compartment, sections from joints 3- to 84 days post-DMM were stained immunohistochemically (IHC) for type II collagen and DAPI-positive cells ($n=5$ -mice/group, see Supplemental Methods), and quantified via custom semi-automated image processing. IHC staining was performed on slides adjacent to the Safranin-O sections, and surrounding the center of cartilage contact (levels 2–4). Following staining, overlapping images of the sections were captured at $20\times$ magnification using an epifluorescent microscope (AxioObserver.Z1, Carl Zeiss, Thornwood, NY) and a digital camera (AxioCam MrC, Zeiss) and combined (tiled) using Zen software (Zeiss). A custom semi-automated MATLAB (MATLAB R2015a, The MathWorks, Natick, MA) algorithm was used to: (i) define the MTP region of interest

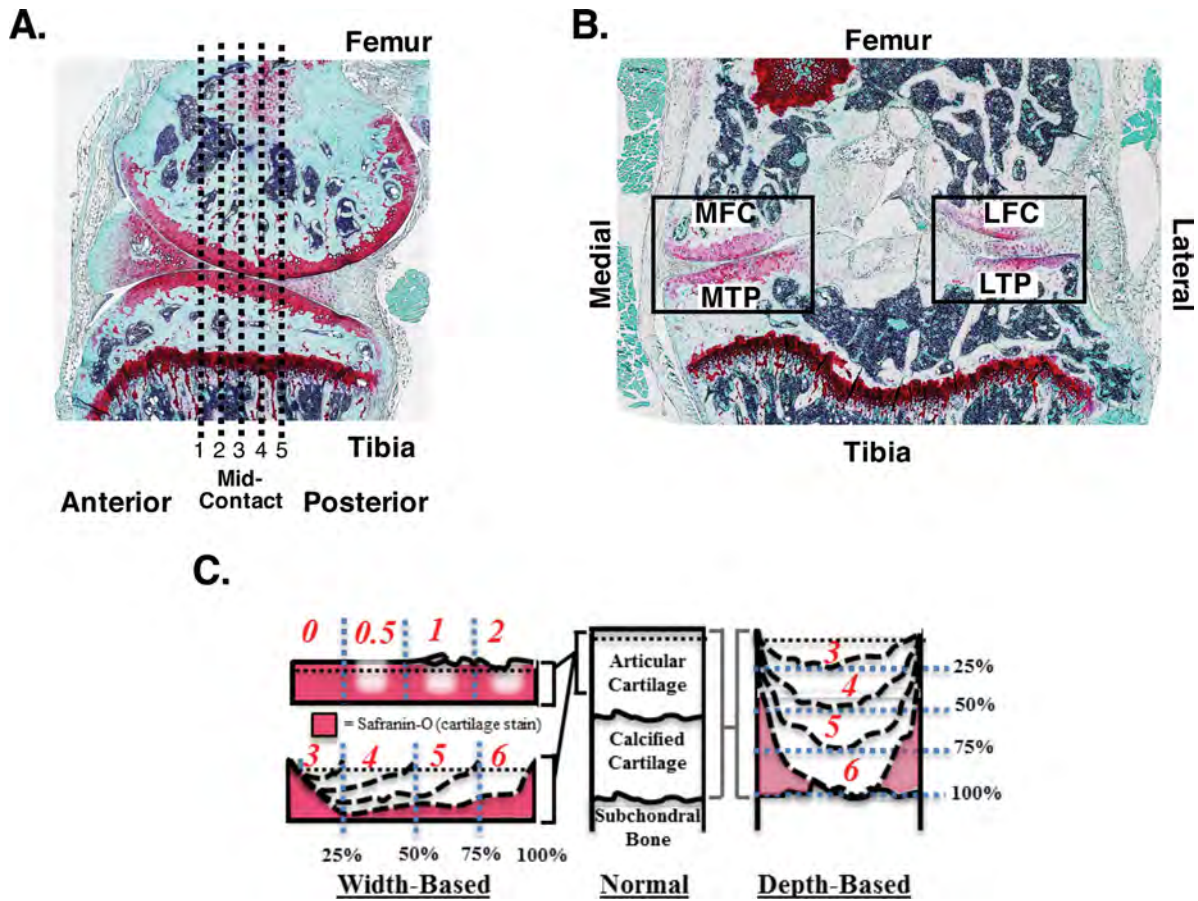


Figure 1. Histological analysis of cartilage structural damage. Representative Safranin-O/fast green stained sagittal (A) section demonstrating the region of interest and slide positions selected for analysis, and (B) a coronal section used for semi-quantitative histological analysis of the medial femoral condyle (MFC), medial tibial plateau (MTP), lateral femoral condyle (LFC), lateral tibial plateau (LTP). Images were captured at 5× magnification. Independent width- and depth-based scoring systems (C) were utilized in this study. Damage values of 0–2 were identical in both scoring systems, while scores of 3 and higher allowed differentiation of width- versus depth-based erosions extending beyond the superficial layer. A complete description of the scoring system can be found in Table 1.

(ROI) in each image; (ii) manually trace the AC, CC, and MM; and (iii) count DAPI-positive cells within the AC and CC (Supplementary Fig. S1). Traces were automatically segmented width-wise into four regions (quadrants 1–4; Supplementary Fig. S1) and cartilage structural (i.e., AC and CC thickness), and cellular (i.e., number of DAPI-positive cells)

parameters were quantified within each quadrant. Quadrant 1 represents the innermost region of the joint while quadrant 4 was nearest the joint margin. Additionally, the degree of meniscal extrusion was calculated as the extent of cartilage surface covered by the meniscus (length of meniscus covering AC/total AC width; herein referred to as “meniscal coverage”),

Table 1. Description of the Semi-Quantitative Width- and Depth-Wise Histological Scoring Systems Utilized in the Present Study

Score	Width-Based	Depth-Based
0	Normal	
0.5	Loss of Safranin-O without structural changes ^a	
1	Fibrillations/discontinuities and/or roughened superficial surface ^a	
2	Erosion to the layer immediately below the superficial layer and some loss of surface lamina ^a	
3	Erosion ^b extending <25% of AC width	Erosion ^b extending for <25% of total cartilage depth ^c
4	Erosion ^b extending 25–50% of AC width	Erosion ^b extending for 25–50% of total cartilage depth ^c
5	Erosion ^b extending 50–75% of AC width	Erosion ^b extending for 50–75% of total cartilage depth ^c
6	Erosion ^b extending >75% of AC width	Erosion ^b extending for >75% of total cartilage depth ^c

^aIndicates damage can extend across any percentage (%) of the articular cartilage surface width.

^bIndicates erosion must extend beyond the layer immediately below the superficial layer/lamina.

^cIndicates depth measured relative to total cartilage (AC + CC) thickness.

and the linear extent of width-wise cartilage surface damage, including fibrillations, clefting, and erosions (length of AC damage/total AC width), were traced,²⁵ and quantified.

Statistical Analysis

All data are presented as mean $\pm$ standard deviation. Statistical analyses were performed using GraphPad Prism 6.0 for Mac (GraphPad Software, La Jolla, CA). Paired *t*-tests were used to establish differences in cartilage outcomes between DMM and contralateral joints at each timepoint. One-way ANOVAs with Tukey's post-hoc tests were used to determine differences in cartilage outcomes (i) between contralateral, sham, and age-matched joints at a given timepoint; and (ii) over time for either DMM or control joints. Linear regression was performed to identify (i) spatial patterns (among levels 1–5) within DMM or contralateral joints; and (ii) the relationship between whole-joint depth- versus width-wise damage. Pearson correlation co-efficients were calculated to establish relationships between meniscal extrusion/coverage, chondrocyte loss, and cartilage damage. Statistical significance and trends were set at $p < 0.05$ and $p < 0.10$, respectively.

RESULTS

Whole-Joint Temporal Changes in AC Structure Post-DMM

Early and progressive changes in AC structure at the whole-joint level were confirmed within the medial compartments of DMM joints (Fig. 2 and Supplemental Table S1). Within the MFC, DMM joints exhibited a trend toward increased whole-joint cartilage damage (i.e., loss of proteoglycan staining and the presence of fibrillations/clefting) relative to contralateral limbs as early as 7 days (Fig. 2A and B), and significant damage accumulation beyond 56 days. In the MTP, similar damage progression was observed in the DMM joints; however, this damage was statistically significant as early as 3-days (score = 0.7; $p < 0.05$; Fig. 2C and D). In both the MFC and MTP of DMM

joints, damage appeared to stabilize (scores of ~ 1 –2) between 7- and 56 days before increasing again at 84 days and beyond (scores ≥ 3 ; $p < 0.001$). At 112 days, damage scores highlighted a moderate degree of width- and depth-wise cartilage erosion (~ 25 –50% of the MTP width involved and $\sim 50\%$ of the AC depth; scores = 3.3 and 3.4, respectively). No significant cartilage damage was observed within the lateral compartments of DMM compared to contralateral joints or within contralateral joints compared to sham, age-matched, and non-surgical controls (Supplemental Table S1).

We also investigated whether the use of semi-quantitative scoring of width- or depth-wise erosion resulted in different interpretations of AC damage progression by plotting MFC and MTP width-wise scores against their paired depth-wise scores and performing linear regression (Supplemental Fig. S2). Damage scores ≤ 2 are classified identically in both schemes, and thus, exhibit an expected one-to-one relationship (MFC [slope = 0.98; $r^2 = 0.99$] and MTP [slope = 1.0; $r^2 = 0.99$]). However, for scores > 2 the slope of the linear regression differed significantly from 1.0 in the MTP (slope = 0.75; $r^2 = 0.79$), indicating a slight, albeit significant preference for higher width-wise damage scores. A trend toward significance was observed in scores > 2 in the MFC (slope = 0.83; $r^2 = 0.86$). However, the overall interpretation of damage progression remained similar (Fig. 2).

Anterior-to-Posterior Distribution of Cartilage Damage Post-DMM

To determine the anterior-to-posterior progression of cartilage damage within the joint, we investigated the distribution of semi-quantitative histological scores

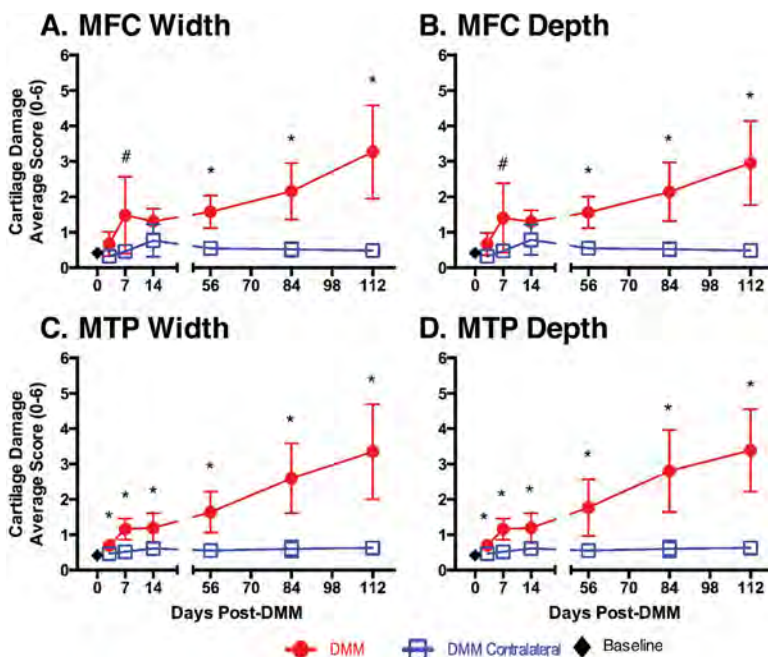


Figure 2. Progression of whole-joint cartilage damage post-DMM. Semi-quantitative histological scoring of width- (A) and depth-wise (B) MFC damage, and width- (C) and depth-wise (D) MTP damage across the joint demonstrated an early accumulation of proteoglycan loss and surface fibrillations post-DMM (scores = 0.5–1 by 3 days), which preceded surface clefting (scores = 1–2 at 7- to 56 days) and overt cartilage erosions (scores ≥ 3 at 84+ days). Results are presented as mean $\pm$ STD ($n = 5$ –10/timepoint/group) where * $p < 0.05$, # $p < 0.10$ (trend) for paired *t*-test between DMM and DMM contralateral.

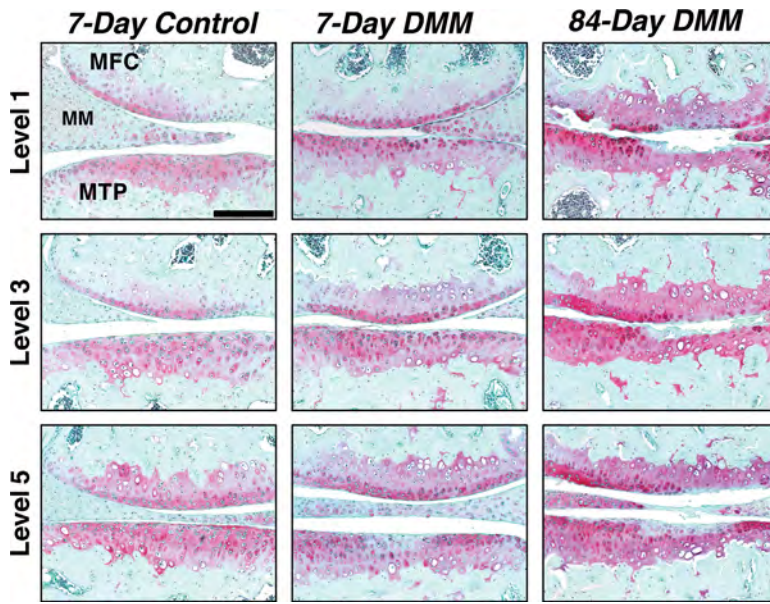


Figure 3. Spatiotemporal progression of cartilage damage in medial knee compartments post-DMM. Representative Safranin-O/fast green stained sections highlight the changes in medial meniscus (MM) location and the distribution of cartilage damage from the anterior (level 1) to posterior (level 5) of DMM and contralateral (control) joints. Anteriorly localized cartilage damage was qualitatively observed in the MTP of DMM joints at later timepoints. Images were acquired at 20 $\times$ magnification, scale bar (200- μ m) applies to all images.

across the medial compartment from level 1 (anterior) to level 5 (posterior) over time. Our qualitative impression of preferential, anteriorly localized MTP damage (Fig. 3) was confirmed by semi-quantitative analysis of damage distribution among Safranin-O stained sections (Fig. 4). In the MFC of DMM joints, no significant spatial preference was observed at any timepoint (width-wise Fig. 4A; depth-wise Supplementary

Fig. S3). In contrast, anteriorly localized damage was observed in the MTP at later-stage disease (width-wise damage $p = 0.02$ and 0.08 at 84- and 112 days, respectively, Fig. 4B; depth-wise Supplementary Fig. S3).

Meniscal Coverage of the AC Surface Post-DMM

Qualitative observations in both Safranin-O and IHC stained sections (Fig. 5) reinforced our impressions of

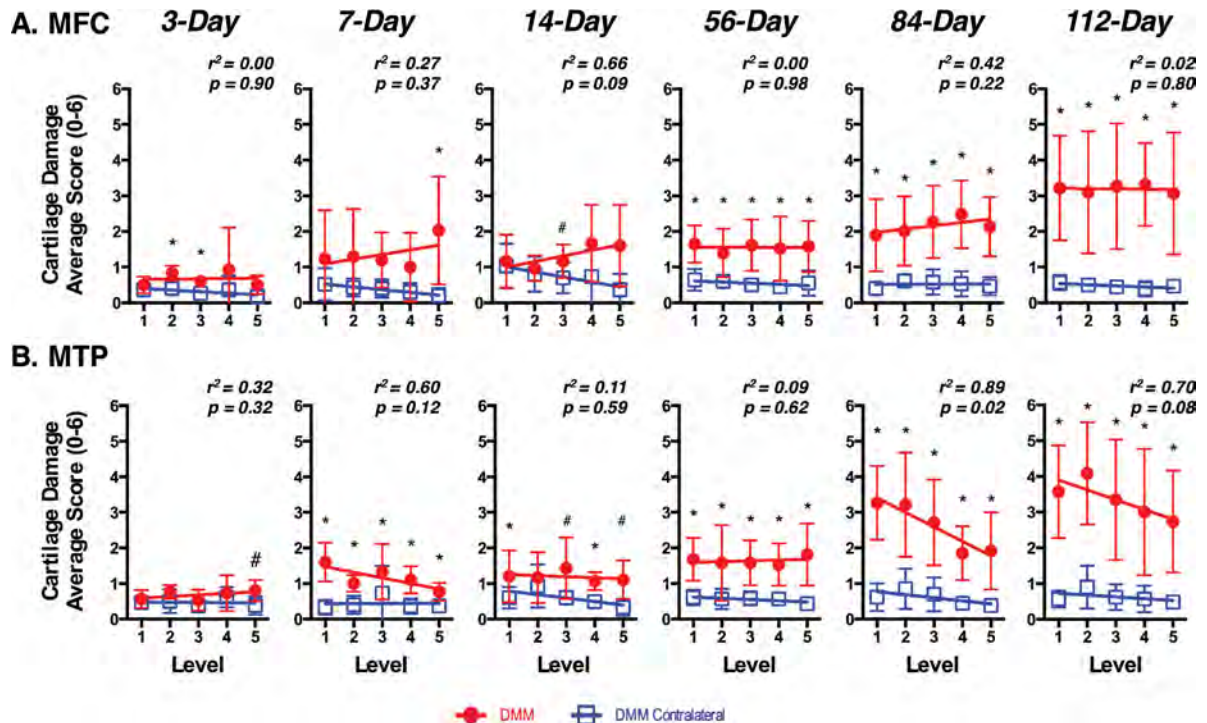


Figure 4. Anterior-to-posterior distribution of width-wise cartilage damage within the medial knee compartments post-DMM. Semi-quantitative width-wise scoring of cartilage damage in the MFC (A) and MTP (B), from anterior (level 1) to posterior (level 5) of the joint. The appearance of spatially dependent damage was only observed in the MTP of DMM joints at late timepoints (84- and 112 days), i.e., increased damage anteriorly compared to posteriorly. Results are presented as mean $\pm$ STD ($n = 5-10$ /timepoint/group) where * $p < 0.05$ and # $p < 0.10$ (trend) for paired t -test between DMM and contralateral joints at a given level. Linear regression r^2 - and p values are shown for DMM joints only.

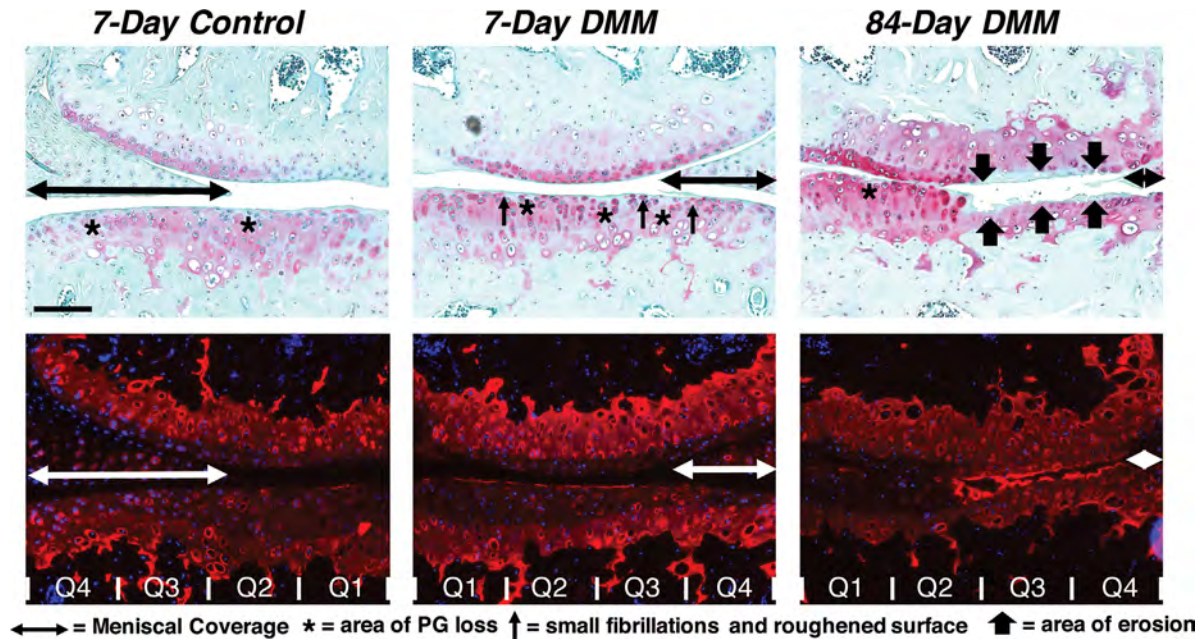


Figure 5. Histological and immunohistochemical demonstration of the spatial distribution (medial-to-lateral) of cartilage damage, meniscal coverage, and the presence of chondrocytes post-DMM. Adjacent histological (Safranin-O/fast green, upper panel) and immunohistochemical (type II collagen (red) and DAPI (blue), lower pane) images at 7- and 84 days post-DMM. A distinct loss of proteoglycan staining and chondrocyte presence, as well as increased type II collagen staining was observed to co-localize to MTP cartilage regions that experienced a loss of meniscal coverage at 7- and 84 days (Quadrant 3 [Q3]). All sections depict images taken from anterior-posterior level 2 (anterior-to-mid-contact) and acquired at 20 $\times$ magnification, scale bar (100- μ m) applies to all images.

a spatial relationship between AC damage in the joint and meniscal extrusion post-DMM. IHC sections (type II collagen) were used to measure the extent of the central MTP (levels 2–4) covered by the MM. In non-surgical controls and contralateral limbs, $\sim 50\%$ of the MTP was covered by the MM throughout the study (Fig. 6). In contrast, an immediate $\sim 30\text{--}40\%$ reduction in meniscal coverage of the MTP was seen at 3- and 7 days ($p = 0.16$ and 0.05 , respectively). MTP coverage stabilized at $\sim 20\%$ coverage (60% reduction compared to contralateral joints; $p = 0.001$) by 56 days. As a result of meniscal extrusion, a full quarter of the central contact region of the MTP (and MFC), largely consisting of quadrant 3 (Q3), experienced an acute and persistent change in meniscal coverage.

Distribution of Width-Wise MTP AC Damage Post-DMM

To assess the width-wise extent of surface damage and erosions not captured by our semi-quantitative scoring system, we quantitated the linear width-wise surface damage, which included fibrillations (score of 1), clefting (2), and erosions (≥ 3), across the MTP in IHC stained sections. We observed that the extent of damaged surface increased from $\sim 0\%$ to 35% of the MTP cartilage width between 0- and 7 days (Fig. 7A), then stabilized at $\sim 35\text{--}40\%$ for the remainder of the study. We also observed that the extent of compromised cartilage surface was inversely correlated with the extent of meniscal coverage ($R^2 = 0.40$ and $p < 0.001$; Fig. 7B) and that the spatial location of this damage was typically centered about the newly exposed Q3 of the MTP (data not shown). In the same

sections, we measured the average thickness of the AC and CC within each quadrant (Q1–4). Interestingly, a statistically significant reduction in AC thickness was only observed within Q3 at 84 days (Supplementary Fig. S4). No change in CC thickness was observed in DMM or contralateral joints (data not shown). We also qualitatively observed that the linear extent of width-wise surface damage coincided with an increase in type II collagen staining in DMM and contralateral joints (Fig. 5). Collagen staining intensity appeared

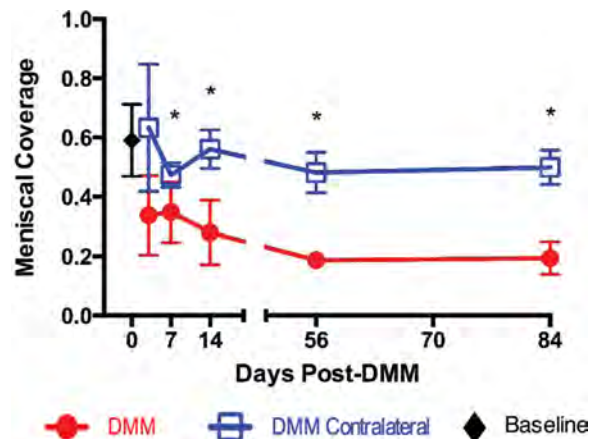


Figure 6. Changes in the degree of the articular cartilage covered by the medial meniscus (meniscal coverage) following DMM. The MTP AC in DMM joints experienced a significant loss of meniscal coverage as early as 7 days following injury; coverage stabilized at $\sim 20\text{--}40\%$ of the AC over time. Results are presented as mean $\pm$ STD ($n = 5/\text{timepoint}/\text{group}$) where $*p < 0.05$ for paired t -test between DMM and contralateral joints.

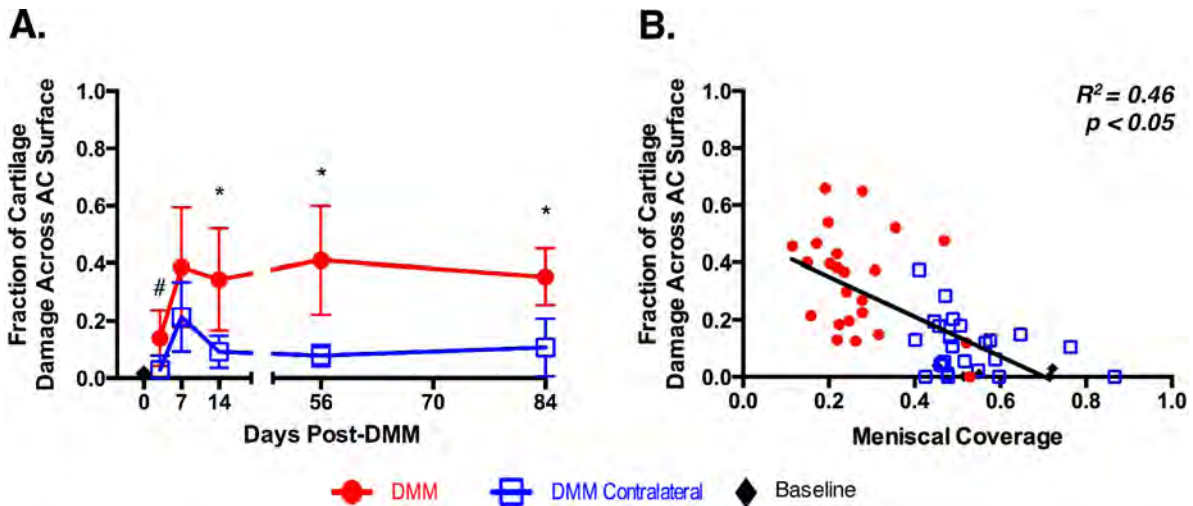


Figure 7. Progression of the degree of surface damage across the width of the MTP post-DMM and its relationship to meniscal coverage. In DMM joints, damage across the width of the MTP surface appeared rapidly following DMM, ultimately stabilizing at ~35–40% involvement of the MTP width over time (A). Results are presented as mean $\pm$ STD ($n = 5$), where * $p < 0.05$ and # $p < 0.10$ (trend) for paired t -test between DMM and contralateral joints. The extent of damage across the width of the MTP was inversely related to the degree of meniscal coverage (B). Pearson correlation co-efficients (R^2)- and p -values indicated.

greater at all timepoints in DMM joints, while also appearing to co-localize to AC regions demonstrating a loss of Safranin-O staining, and later overt damage.

Spatiotemporal Changes in MTP Chondrocyte Cellularity Post-DMM

Lastly, in analyzing IHC stained sections we observed alterations in the spatial distributions of chondrocytes (DAPI-positive cells) within the AC post-DMM (Fig. 5). As early as 3 days, a significant loss of chondrocytes was seen in Q3 of the AC of DMM joints (~60% reduction compared to contralateral joints, $p = 0.005$; Fig. 8A), which progressed until that quadrant was effectively devoid of chondrocytes by 84 days. In Q1, chondrocyte number remained unchanged, while Q2 and Q4 exhibited less substantial chondrocyte losses (Fig. 8A). In contralateral joints, no significant changes in chondrocyte number were observed. Furthermore, we observed that the number of AC chondrocytes in quadrants 2, 3, and 4 were linearly correlated to the degree of meniscal coverage (Fig. 8B). No significant correlation between meniscal coverage and chondrocyte number was observed in Q1. The number of chondrocyte in MTP CC remained unaltered (data not shown).

DISCUSSION

In the present study, we identified early, localized chondrocyte loss and cartilage damage in the murine DMM model of PTOA using semi-quantitative histological scoring and quantitative analysis. The findings of immediate cellular, compositional, and structural changes to cartilage highlighted the rapidity of the post-DMM injury response in male C57BL/6 mice and refined the timeline of damage initiation following meniscal destabilization. While our findings were consistent with previous semi-quantitative studies of

DMM in the mouse,^{4–22,24,29} rat,³¹ and rabbit,³⁰ and with early clinical changes seen in human cartilage following ACL tears,³⁴ we also observed that semi-quantitative cartilage scoring schemes, whether based upon width- or depth-wise classifications, did not provide a complete picture of the progression of cartilage degeneration. Instead, through implementation of detailed spatial analysis of cartilage damage progression, we uncovered additional aspects of the post-DMM cartilage injury response. Specifically, a strong relationship between meniscal extrusion/coverage and the location/progression of AC cellularity and structural changes.

Under physiological conditions, the meniscus stabilizes the joint and transmits load during articulation.³⁵ After meniscal detachment from the tibia, either from natural root tears or surgical procedures (like DMM), the meniscus' stabilizing function is lost as wedging effects³⁵ between the femoral condyle and meniscus causes its extrusion into the joint space. As a result of this alteration, several joint-level changes occur, including increased joint laxity and anterior motion,²⁹ and increased tibial plateau contact stresses.^{10,30} Clinically, increased meniscal extrusion has been related to increased cartilage damage.^{36–38} Our findings in the murine DMM model are consistent with these observations. We observed that DMM-induced meniscal extrusion led to an immediate alteration in meniscal coverage of the MTP AC surface, and the co-localization of cartilage changes (e.g., loss of chondrocytes and proteoglycan content, surface damage, and overt erosions) to the uncovered regions. These findings confirmed the outward extrusion of the MM within the joint post-DMM, and identified the specific regions of the AC that are exposed to potentially altered contacts and mechanics due to changes in meniscal coverage.

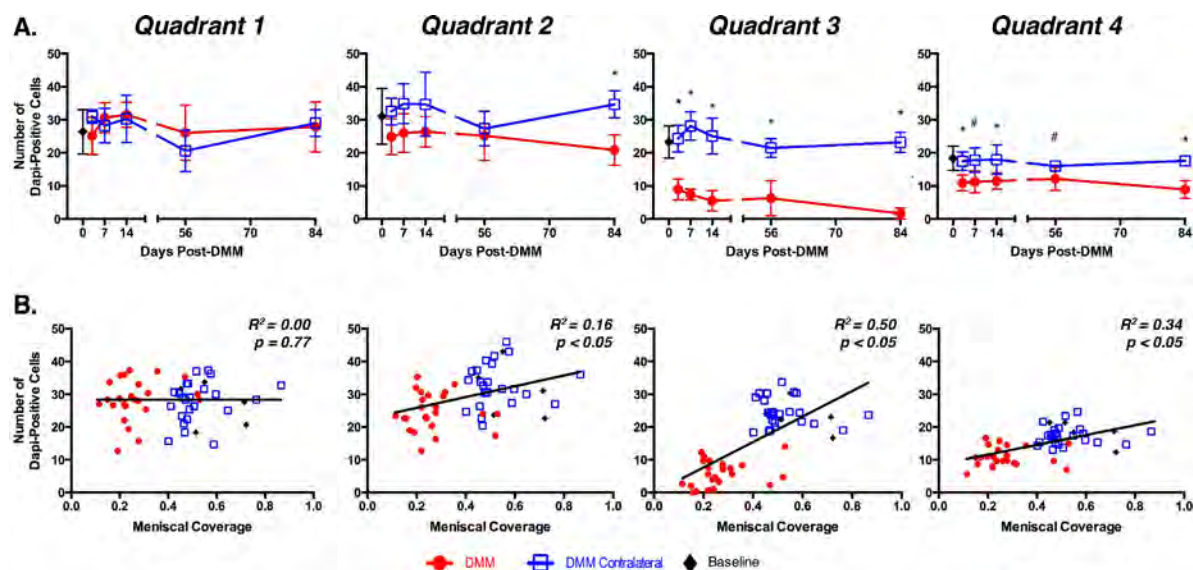


Figure 8. Spatiotemporal quantification of chondrocyte presence across the MTP of articular cartilage post-DMM and their relationship to meniscal coverage. The MTP demonstrated a significant loss of chondrocytes that localized to specific MTP quadrants (Q2–4) (A) that also experienced alterations in meniscal coverage. In these quadrants, chondrocyte loss was linearly related to degree of meniscal coverage (B). Results are presented as mean $\pm$ STD ($n = 5/\text{timepoint/group}$) where * $p < 0.05$ and # $p < 0.1$ (trend) for paired t -test between DMM and DMM contralateral. Pearson correlation co-efficients (R^2)- and p -values indicated.

Through the detailed spatiotemporal analysis of cartilage damage, the present study provides insight into the anterior-to-posterior and medial-to-lateral progression of cartilage damage post-DMM. While the present analysis, demonstrating a progressive increase in anteriorly localized MTP damage, was limited to coronal sections spanning the central-cartilage-contact-region of each joint, and thus, may exclude damage more anteriorly or posteriorly, our results were consistent with similar observations made by Glasson et al., in the initial murine DMM study.³ Analysis of a small set of sagittally sectioned joints confirmed our findings across the full MTP, while also suggesting an increase in damage in the MFC anteriorly (Fig S5). This was not evident in the semi-quantitative analysis, potentially due to increased variability within femoral cartilage scoring.²⁸ Additionally, the sagittal sections from joints 3 days post-DMM demonstrated, similar to the Glasson study,³ no signs of DMM inducing inadvertent cartilage damage. Interpreting these results in light of established joint-level biomechanical changes^{10,29,30} supports the hypothesis that DMM-mediated meniscal extrusion causes altered anterior-to-posterior loading of the knee joint and subsequent anteriorly-to-centrally localized cartilage damage.

A distinct and informative pattern of medial-to-lateral damage progression in the medial joint post-DMM was also observed. Damage across the width of the MTP surface accumulated rapidly and then stabilized, resulting in the presence of increased type II collagen staining and extensive fibrillation, and clefting across ~ 20 – 40% of the MTP surface by 7 days, with this damage being localized to MTP regions experiencing alterations in meniscal coverage due to

meniscal extrusion. Interestingly, as time progressed (84+ days) cartilage erosions and a decrease in AC thickness also co-localized to the same MTP regions. Similar findings were observed for the MFC (data not shown). The highly focal nature of this damage highlights the important role of meniscal extrusion/coverage in establishing the location of damage initiation and progression in the DMM model. While this study does not identify the cause of this focal damage progression, several mechanisms could be involved including: (i) alterations in cartilage-on-cartilage and cartilage-on-meniscus contact mechanics^{29,30} following extrusion; (ii) differences in the mechanical properties of cartilage tissues previously covered by the meniscus^{39–41}; and/or (iii) changes in the accessibility of inflammatory⁴² and pro-catabolic mediators to the local cartilage regions post-injury. Determining if these, or other, mechanisms are involved in the observed phenomena requires further investigation. The apparent stabilization of AC surface fibrillation and clefting between 7- and 56 days post-DMM was interesting and consistent with previous studies.⁴ This may reflect a natural ability of the cartilage extracellular matrix to withstand alterations in the tissue's mechanical environment and even loss of resident chondrocytes, temporarily, before developing into larger erosions at later disease stages.

A potentially more significant finding with regards to understanding PTOA initiation was the identification of a striking, immediate, and highly focal loss of chondrocytes within the AC of injured joints post-DMM. As early as 3 days, a drastic decrease in chondrocyte number was observed within the regions of the MTP that experienced changes in meniscal coverage (Q3). Over time, chondrocyte number continued to

decrease in DMM joints compared to their contralateral joints until there was a complete loss of cells in Q3, and a moderate loss of chondrocytes in adjacent quadrants. Given the critical role that chondrocytes play in the maintenance, repair, and regeneration of AC,⁴³ this rapid and focal loss of chondrocytes may constitute the precipitating biological event leading to the initiation and progression of PTOA. This idea is supported by two observations: (i) that cartilage erosions predominately localize to areas of earlier chondrocyte loss within and surrounding the “uncovered” quadrant (Q3); and (ii) that the innermost MTP quadrant (Q1) appeared more resistant to both chondrocyte loss and cartilage damage. Together, this data suggest that rapid chondrocyte loss post-DMM co-localized to AC quadrants experiencing an immediate loss of meniscal coverage (Q3). Subsequently, progression of cartilage damage (fibrillations, clefts, and erosions) may also be driven by this focal loss of meniscal coverage and chondrocytes.

While the rapid and localized loss of chondrocytes within the AC of DMM joints in the present study was remarkable, the biological mechanisms underlying this pattern of cell loss remains unknown. Numerous studies implicate chondrocyte necrosis and apoptosis in the initiation and progression of cartilage degeneration following severe, acute cartilage injury *in vitro*,^{42,44} and in murine DMM models of PTOA.^{4,6,24,27,33} In a non-invasive murine cartilage injury study, co-localization of chondrocyte apoptosis to specific regions of injurious load application was identified 5 days post-injury³² and a loss of DAPI-positive cells was observed at 14 days, consistent with the present study. The early and rapid loss of chondrocytes in our study also indicate that significant changes in chondrocyte health occur very quickly, prior to 3 days, post-DMM. In this regard, a recent study by Burleigh et al.¹¹ identified changes in gene expressions within murine cartilage as early as 6 h post-DMM. Further studies are required to establish: (i) whether the observed loss of chondrocytes in DMM joints is attributable to apoptotic or necrotic events; (ii) when, relative to the timing of injury, such critical biological changes are initiated; and (iii) the molecular and cellular mechanisms that precipitate these changes.

Of important note is that the present study focused on the role of DMM-induced meniscal extrusion on the spatiotemporal of cartilage changes in male C57BL/6 mice due to the pervasive use of the DMM model^{4–25,29,45,46} and the increased susceptibility of these mice to develop cartilage damage in this model.²³ Whether similar outcomes would be observable in female C57BL/6 mice,²³ other mouse strains,³ species,^{30,31} destabilizing injury models,^{32,47,48} or clinical injuries requires further investigation. However, given the similarities in the progression of cartilage damage among the murine DMM and other injury models, we suspect that the observed relationships may be a general feature of

PTOA initiation and progression. Although this response pertains to the murine DMM model, we believe these data support the expanded study of immediate restoration of meniscal function for providing chondroprotective benefit post-injury, both pre-clinically and clinically.

It is important to acknowledge a few limitations of this study. Firstly, histological-based approaches may introduce artifacts in the analysis of meniscal extrusion due to variability in knee joint positioning during fixation, embedding, and sectioning (we attempted to place all joints in the same naturally flexed position, however, some section orientation variability was observed). However, given the small deviations in meniscal positioning that we observed, as well as the robust correlations between damage degree, location, and meniscal coverage, we are confident that our data reflect a strong relationship between meniscal extrusion/coverage and AC changes. Secondly, our analysis does not provide insight into the spatial evolution of proteoglycan loss, which may have significant impact on early cartilage biomechanical functionality. While we qualitatively noted early proteoglycan loss in Q3 of the MTP, the relationship between proteoglycan depletion and chondrocyte loss remains unknown. Lastly, while this study incorporated independent mechanisms for width- and depth-wise semi-quantitative scoring of cartilage erosions, we found depth-wise scoring harder to evaluate due to the variability in the position of the CC/subchondral bone interface across the MTP, as well as the fact that erosion of the AC made determination of relative depth-wise damage increasingly difficult. However, given the general similarities in width- and depth-wise damage progression, we suggest, for the sake of simplicity and unambiguity, using width-wise scoring for evaluating mouse cartilage damage.

Finally, the present findings have several implications regarding the study of PTOA prevention and treatment in the DMM model and translational studies. The focal population of cells identified represent an ideal target to investigate the chondroprotective effects of various pre-clinical therapies, including joint unloading/rehabilitation¹¹ and pharmacological interventions.^{12,14,17,20,22} However, it appears that such interventions may need to be initiated immediately/shortly post-injury to have the greatest anti-osteoarthritic potential, an observation that may explain the equivocal findings of chondroprotective drugs within clinical trials.⁴⁹ Alternatively, the presence of an apparent plateau in the progression of cartilage damage, between ~7–56 days post-DMM, supports the possibility of the DMM-model as a platform to test alternative treatment targets based on cartilage regeneration.⁵⁰

In conclusion, the approaches described here present a benchmark for the detailed study of cartilage damage progression within murine models

of surgery-/injury-induced PTOA while providing detailed analyses of the spatiotemporal progression and relationships between cartilage damage, changes in chondrocyte cellularity, and meniscal extrusion within the murine DMM model. This study also provides a comprehensive baseline for ongoing assessment of the cellular and molecular pathoetiology of PTOA, as well as the study of its prevention and treatment.

AUTHORS' CONTRIBUTIONS

All authors have read and approved the final submission of the manuscript. The contribution of the authors are described below: Study design—MAD, MKS, CP. Animal research—MAD, MKS. Acquisition of data—MAD, MKS, RNP, ATW, RCL. Analysis and interpretation—MAD, CP. Manuscript preparation—MAD, MKS, CP. Statistical analysis—MAD.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's website.

Supplementary Methods:

Immunohistochemical Staining: Sections from DMM and contralateral (control) joints were stained immunohistochemically for type II collagen and the presence of cells (DAPI counterstain for nuclei). Type II collagen was used as a structural indicator to assist in distinguishing between the articular and calcified cartilages within each section and DAPI counterstain was used to differentiate the presence of chondrocytes in each tissue. Briefly, antigen retrieval was performed on the sections using pepsin (0.4% w/v; Sigma) in 0.01N hydrochloric acid for 15 minutes at 37°C. Sections were then blocked with 5% normal donkey serum (Sigma) in phosphate-buffered saline with 0.1% Tween-20 (Fisher Scientific) for one-hour at room temperature (RT), and incubated with a rabbit anti-mouse type II collagen primary antibody (1:200 dilution, ThermoFisher) overnight at 4°C. Sections were then incubated with an AlexaFluor-555 donkey anti-rabbit secondary antibody (1:400, ThermoFisher) for 1 hour at RT in the dark, treated with 0.1% w/v Sudan black (FisherBioReagents™) in 70% ethanol for 30 minutes at RT to reduce autofluorescence, and then mounted with a DAPI-containing mounting medium (ThermoFisher) for the detection of cell nuclei. Fluorescent images of the collagen type II and DAPI stained sections were obtained within 72-hours of completing the immunohistochemical staining procedure to limit fluorescent signal loss.

Supplemental Table 1: Temporal quantification of cartilage damage scoring in DMM, Contralateral, Sham and Age-Matched Joints

Days Post-Surgery	Medial Femoral Condyle (Width-Wise Score)				Lateral Femoral Condyle (Width-Wise Score)			
	DMM	Contralateral	Sham	Age-Matched [‡]	DMM	DMM Contralateral	Sham	Age-Matched [‡]
3	0.67 ± 0.34	0.33 ± 0.07	0.47 ± 0.20	0.42 ± 0.14	0.55 ± 0.14	0.65 ± 0.28	0.47 ± 0.15	0.39 ± 0.18
7	1.48 ± 1.09	0.46 ± 0.14	0.52 ± 0.11	"	0.71 ± 0.30	0.51 ± 0.27	0.70 ± 0.17	"
14	1.31 ± 0.35	0.77 ± 0.46	0.51 ± 0.08	"	0.63 ± 0.14	0.69 ± 0.36	0.44 ± 0.19	"
56	1.58 ± 0.46***	0.55 ± 0.12	0.41 ± 0.08	0.49 ± 0.27	0.66 ± 0.30	0.70 ± 0.24	0.50 ± 0.05	0.55 ± 0.34
84	2.16 ± 0.80****	0.52 ± 0.19	0.56 ± 0.22	0.47 ± 0.06	0.81 ± 0.35	0.71 ± 0.21	0.83 ± 0.20	0.67 ± 0.19
112	3.27 ± 1.32****	0.49 ± 0.07	0.38 ± 0.02	0.40 ± 0.14	1.34 ± 0.49	1.20 ± 0.46	0.73 ± 0.15	1.16 ± 0.65
Days Post-Surgery	Medial Femoral Condyle (Depth-Wise Score)				Lateral Femoral Condyle (Depth-Wise Score)			
	DMM	DMM Contralateral	Sham	Age-Matched [‡]	DMM	DMM Contralateral	Sham	Age-Matched [‡]
3	0.67 ± 0.32	0.32 ± 0.07	0.47 ± 0.20	0.42 ± 0.14	0.55 ± 0.14	0.65 ± 0.28	0.47 ± 0.15	0.39 ± 0.18
7	1.41 ± 0.98	0.47 ± 0.13	0.52 ± 0.11	"	0.71 ± 0.30	0.53 ± 0.32	0.70 ± 0.17	"
14	1.30 ± 0.33	0.79 ± 0.43	0.51 ± 0.08	"	0.63 ± 0.14	0.68 ± 0.35	0.44 ± 0.19	"
56	1.57 ± 0.45***	0.55 ± 0.12	0.41 ± 0.08	0.49 ± 0.27	0.64 ± 0.25	0.69 ± 0.21	0.43 ± 0.12	0.55 ± 0.34
84	2.14 ± 0.83****	0.52 ± 0.19	0.56 ± 0.22	0.47 ± 0.06	0.79 ± 0.31	0.71 ± 0.21	0.83 ± 0.20	0.67 ± 0.19
112	2.95 ± 1.18***	0.49 ± 0.08	0.38 ± 0.02	0.41 ± 0.15	1.38 ± 0.55	1.18 ± 0.43	0.73 ± 0.15	1.04 ± 0.51
Days Post-Surgery	Medial Tibia Plateau (Width-Wise Score)				Lateral Tibia Plateau (Width-Wise Score)			
	DMM	DMM Contralateral	Sham	Age-Matched [‡]	DMM	DMM Contralateral	Sham	Age-Matched [‡]
3	0.70 ± 0.16*	0.47 ± 0.06 ^S	0.73 ± 0.03 ^{C,AM}	0.42 ± 0.18 ^S	0.70 ± 0.13	0.69 ± 0.19	0.83 ± 0.22	0.51 ± 0.24
7	1.16 ± 0.30*	0.52 ± 0.14	0.59 ± 0.05	"	0.88 ± 0.29	0.77 ± 0.25	0.92 ± 0.21	"
14	1.19 ± 0.42*	0.61 ± 0.16	0.65 ± 0.06	"	0.78 ± 0.20	0.75 ± 0.16	0.57 ± 0.04	"
56	1.64 ± 0.58***	0.55 ± 0.10	0.63 ± 0.17	0.56 ± 0.31	0.93 ± 0.22	0.76 ± 0.23	0.77 ± 0.12	0.68 ± 0.26
84	2.59 ± 0.98****	0.60 ± 0.24	0.61 ± 0.15	0.63 ± 0.15	0.98 ± 0.34	1.01 ± 0.34	1.10 ± 0.31	0.80 ± 0.11
112	3.33 ± 1.35****	0.63 ± 0.18	0.86 ± 0.01	0.74 ± 0.37	1.07 ± 0.34	1.03 ± 0.37	0.96 ± 0.12	0.74 ± 0.21
Days Post-Surgery	Medial Tibia Plateau (Depth-Wise Score)				Lateral Tibia Plateau (Depth-Wise Score)			
	DMM	DMM Contralateral	Sham	Age-Matched [‡]	DMM	DMM Contralateral	Sham	Age-Matched [‡]
3	0.70 ± 0.16*	0.47 ± 0.06 ^S	0.73 ± 0.03 ^{C,AM}	0.42 ± 0.18 ^S	0.70 ± 0.13	0.69 ± 0.19	0.83 ± 0.22	0.51 ± 0.24
7	1.16 ± 0.30*	0.52 ± 0.14	0.59 ± 0.05	"	0.88 ± 0.29	0.73 ± 0.23	0.92 ± 0.21	"
14	1.19 ± 0.42*	0.61 ± 0.16	0.65 ± 0.06	"	0.78 ± 0.20	0.75 ± 0.16	0.58 ± 0.04	"
56	1.77 ± 0.80***	0.55 ± 0.10	0.63 ± 0.17	0.56 ± 0.31	0.93 ± 0.22	0.76 ± 0.23	0.77 ± 0.12	0.68 ± 0.26
84	2.80 ± 1.16****	0.60 ± 0.24	0.61 ± 0.15	0.63 ± 0.15	0.97 ± 0.33	1.00 ± 0.32	1.12 ± 0.33	0.80 ± 0.11
112	3.39 ± 1.17****	0.63 ± 0.18	0.86 ± 0.01	0.74 ± 0.37	1.10 ± 0.36	1.03 ± 0.40	0.96 ± 0.12	0.72 ± 0.21

* = Significantly different from respective contralateral joint, p<0.05; ** = p<0.01; *** = p<0.001; **** = p<0.0001; paired t-test, GraphPad Prism

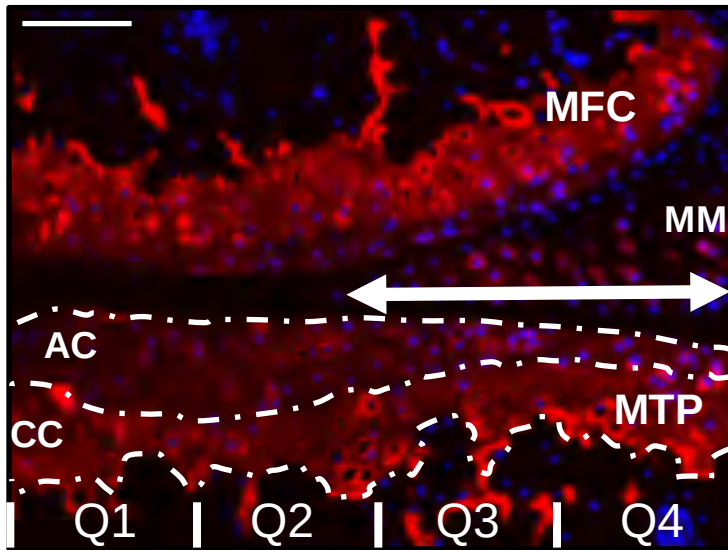
^C = Significantly different from respective contralateral joint, p<0.05; One-way ANOVA, Tukey's post hoc test, GraphPad Prism

^S = Significantly different from respective Sham joint, p<0.05; One-way ANOVA, Tukey's post hoc test, GraphPad Prism

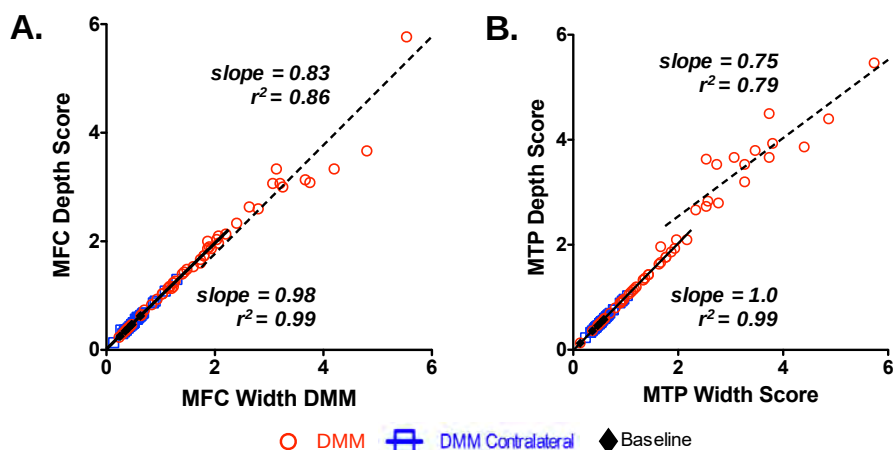
^{AM} = Significantly different from respective age-matched joint, p<0.05; One-way ANOVA, Tukey's post hoc test, GraphPad Prism

[‡] = Baseline (0d) values used for statistical comparison at 3, 7, and 14-days post-DMM

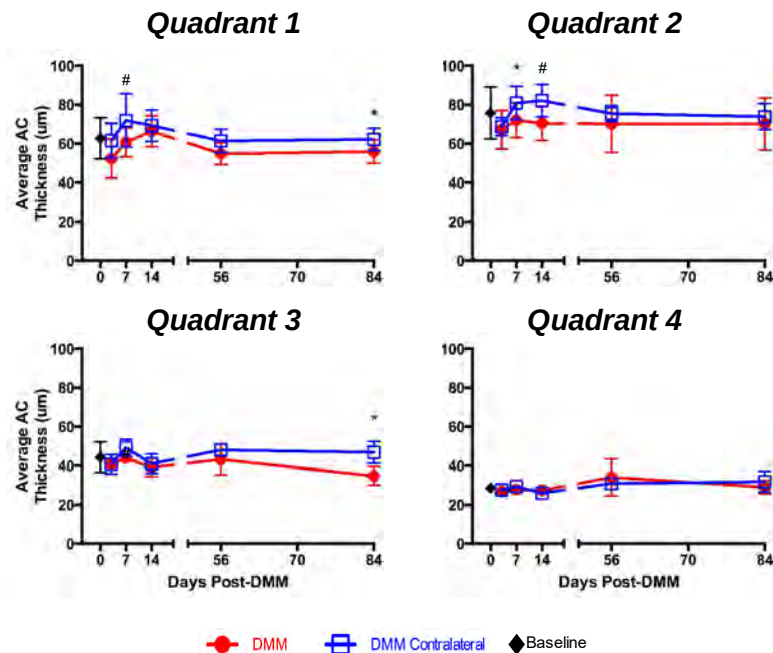
Supplementary Figures:



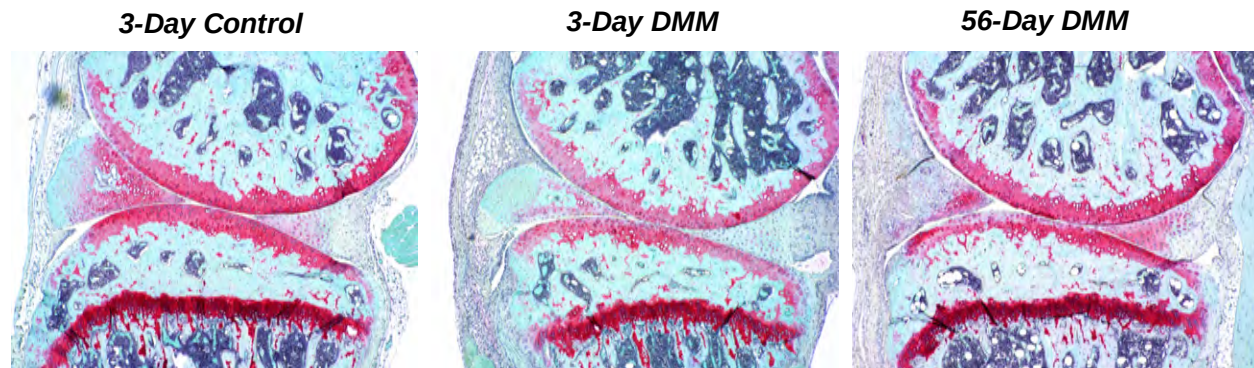
Supplementary Figure 1. Quantification of meniscal coverage, chondrocyte cellularity, and cartilage thickness. Immunohistochemical staining for type II collagen (red; an indicator of cartilage composition) and DAPI-nuclear staining (blue; indicating chondrocytes presence) was performed on sections spanning the central cartilage contact (level 2-4) of the medial joint. The articular cartilage (AC) and calcified cartilage (CC) of the medial tibial plateau (MTP; dotted white lines), and medial meniscus (MM) were manually traced, and the total number of DAPI-positive cells were counted in the AC and CC. Cartilage thickness and chondrocyte number was quantified for each of four separate regions, quadrants 1 (Q1) through quadrant 4 (Q4), representing the innermost through outermost regions of the MTP AC, respectively. The degree of meniscal coverage (MM width/AC width; white arrows) was quantified with respect to the MTP joint margin. Lastly, manual tracing of the extent of linear cartilage damage (including histological values of ≥ 1) across the width of the MTP was performed (not shown in image). All analysis was performed in MATLAB. A representative image of a control joint, oriented with the medial joint margin to the right is shown. Image captured at 20x magnification, scale bar = 100 μ m.



Supplementary Figure 2. Pair-wise comparison of width- vs. depth-wise semi-quantitative cartilage damage scoring in the medial joint compartment. To determine if a preference for overall width- vs. depth-wise erosional damage could be observed following DMM, linear regression was performed on paired semi-quantitative MTP and MFC cartilage damage scores. Since damage scores ≤ 2 are identical in both scoring systems, an expected linear regression slope of ~ 1.0 for damage scores ≤ 2 was observed. However, for damage scores > 2 we observed a slight preference towards increased width-wise erosion compared to depth-wise in the MTP, and no preference in the MFC. Linear regression r^2 - and p-values are shown for values ≤ 2 and values > 2 ; data for all groups are included.



Supplementary Figure 4. Quantification of changes in cartilage thickness across the medial tibial plateau post-DMM. The articular cartilage (AC) thickness in each quadrant of the medial tibia plateau (MTP) was quantified, in MATLAB, from traces of the AC in type II collagen stained sections (level 2-4) using a Euclidian distance transform. A statistically significant decrease in AC thickness was only observed within MTP quadrant 3 at 84-days, which confirms the observed cartilage erosions in this quadrant. Results are presented as mean $\pm$ STD ($n = 5/\text{timepoint}/\text{group}$) where $*$ = $p < 0.05$ and $\#$ = $p < 0.1$ (trend) for paired t-test between DMM and contralateral joints.



Supplementary Figure 5. Histological sections demonstrating the anterior-to-posterior distribution of cartilage damage post-DMM. Representative Safranin-O/fast green stained sagittal sections taken from approximately quadrant 3 of DMM injured and contralateral (control) joints. For orientation, the femur is located on the top, the tibia the bottom, the anterior of the joint to the left and posterior to the right. At 56-days post-DMM there is clear anteriorly-localized cartilage damage in the medial tibia plateau and to a lesser extent in the medial femoral condyle. No apparent localization at 3-days is observed in either the condyle or tibial plateau. Additionally, sections at 3-day post-DMM highlight the lack of blunt surgical trauma to the articular cartilage during the DMM procedure. While inadvertent damage could confound our findings, we are confident that such damage is minimal. Lastly, these images show the consistency of our embedding technique to fix all our joints at approximately the same natural flexed position. Images were acquired at 5x magnification.

Title of Abstract: Quantification of Early Structural Joint Changes in a Murine Model of Post-Traumatic Osteoarthritis

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Introduction: Post-traumatic osteoarthritis (PTOA), an accelerated form of OA, results from traumatic joint injury, e.g., ligament tears, and is common in active individuals. ~50% of patients experiencing a ligamentous tear will exhibit OA within 15-years [1]. Currently, preventative treatments for PTOA are lacking, potentially due to a preclinical focus on mid-to-end-stage disease, whereas the initial precipitating changes remain largely unknown [2]. Herein, we present a detailed quantification of joint changes from early through late-stage disease in a murine joint instability model of PTOA, the destabilization of the medial meniscus (DMM) [3].

Materials and Methods: Adult mice (12-wk male C57BL/6J) underwent DMM surgery in the right joints with contralateral serving as an internal control. Surgical and control joints were harvested at baseline (0-), or 3-, 7-, 14-, 28-, 56-, 84- and 112-days post-injury. Samples were immediately fixed, decalcified, embedded in paraffin, and cut to 5- μ m thick coronal sections. To assess PTOA-induced structural damage, mid-joint sections were stained with Safranin-O/Fast Green. Three blinded individuals scored the extent of cartilage damage on tibial plateau and femoral condylar cartilage using a modified, semi-quantitative scoring system, as well as the degree of synovitis and osteophyte formation (medial compartments only) [4,5]. Paired t-tests (significance set at $p < 0.05$) were performed at each time point between DMM joints and contralateral joints.

Results and Discussion: We found significant site-specific cartilage damage in the medial compartments of DMM joints that appeared as early as 7-days post-injury and increased with time (Figure 1A). No significant cartilage damage was observed in the control joints. Furthermore, the development of synovitis (Figure 1B) and osteophyte formation (Figure 1C) appeared as early as 3-days post-injury in the medial compartments of DMM joints. These results suggest that structural changes in the joint, including bone, cartilage, and synovium responses, are initiated earlier than previously appreciated. Thus, studies attempting to prophylactically prevent PTOA initiation and progression may require immediate-early interventions (≤ 7 d in mice) to be most efficacious.

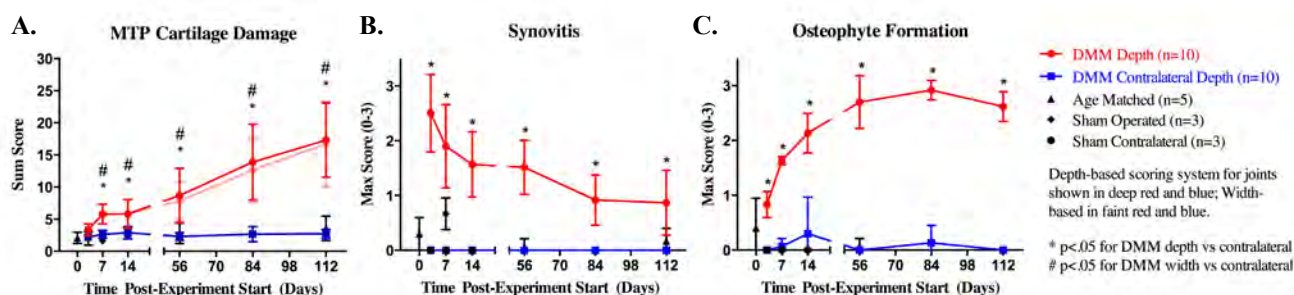


Figure 1. Semi-quantitative scoring of structural changes in the medial compartments of DMM and control joints. Scoring of (A) medial tibia plateau (MTP) cartilage damage, (B) synovitis, and (C) osteophyte formation. Results are represented as mean $\pm$ SD.

Conclusions: Overall, the results of this study demonstrate evidence of significant early (by 7-day) structural changes in the murine DMM model of PTOA, and establish a baseline for the detailed study of the cellular and molecular pathoetiology of PTOA and its prevention through the use of novel, targeted, prophylactic chondroprotective therapeutics.

Acknowledgements: This research was funded by DoD PR120788P1. Special thanks to Brianna Gietter and Fiona Flowerhill for their assistance in this project.

References: [1] Gillquist, J., Sports Med, 1999, 27.3, 143-156; [2] Anderson, D. D., JOR, 2011, 29.6, 802-809 [3] Glasson, S.S., Osteoarthritis Cart, 2007, 15.9,1061-1069; [4] Glasson, S.S., Osteoarthritis Cart, 2010, 18, S17-S23; [5] Chambers, M.G., Arth Rheum, 2001, 44.6, 1455-1465.

Depth- vs. Width-Wise Quantification of Cartilage Damage Following Joint Destabilizing Surgery in the Mouse

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Introduction: Post-traumatic Osteoarthritis (PTOA) is the rapid degeneration of the articular cartilage that often follows serious joint injury, *e.g.*, ruptured ligament or torn menisci. Historically, two semi-quantitative scoring systems (width- or depth-based) have been utilized for ranking the extent of cartilage damage in various animal models of PTOA^{3,5}. Although both methods provide useful information on the etiology of PTOA, these methods have never been used in combination. Therefore, to fully characterize when and how cartilage damage occurs, we utilized and compared both of these scoring systems from early through late stage disease progression in a murine model of PTOA, the destabilization of medial meniscus (DMM). We hypothesized that combining both scoring systems would provide a more complete understanding of how articular cartilage damage proceeds, either widespread or focally, following joint injury.

Methods: DMM surgery was performed on the right knee of 12-week-old male C57BL/6J mice⁴; contralateral joints served as controls. Joints were harvested 0, 3, 7, 14, 56, 84, and 112-days post-surgery. Upon harvest, joints were fixed, decalcified, embedded in paraffin, and sectioned into 5- μ m coronal slices. PTOA-related cartilage damage was assessed by staining 5 equally spaced sections surrounding the region of cartilage-cartilage contact (~500- μ m wide) with Safranin-O/Fast Green. Three blinded individuals scored, using a 0-6 scale, the width- and depth-wise (Figure 1A) extent of cartilage damage in 4 quadrants of the joints: the medial femoral condyle (MFC), lateral femoral condyle (LFC), medial tibial plateau (MTP), and lateral tibial plateau (LTP)^{3,5}. Values were averaged across scorers, and a pair-wise calculation of the ratios of each joints' depth vs. width score were calculated for joints with scores > 2. One sample student t-tests (significance set at $p < 0.05$) were used to compare the ratios for each compartment to a value of 1, which signifies no preference for depth or width-wise damage.

Results: We found previously that cartilage damage increased in a time-dependent manner from early (3-7- days) to late (86-112-days) stage PTOA only in the medial compartments of the DMM joints (Figure 1B)⁶. Reanalyzing this data, at early time points differences among scoring systems are not present (values < 2 are equivalent). However, when comparing the ratio of individual DMM joints' depth and width scores greater than 2, the MTP ratios were significantly greater than 1 indicating a preference toward focal, as opposed to widespread, cartilage damage in the MTP (Figure 1C).

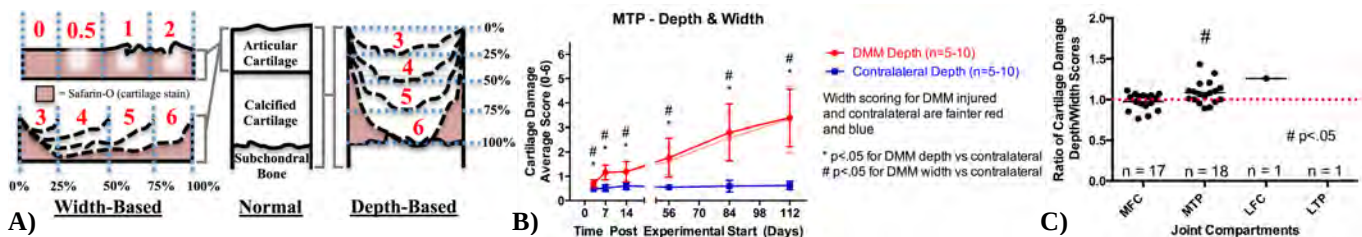


Figure 1: A) Combined scoring system B) Time vs. cartilage damage (MTP) C) Depth vs. width damage (four quadrants)

Discussion: While the two different scoring methods are informative on their own, implementing them together within our modified scoring system allowed for a more holistic view of the natural progression of focal versus widespread cartilage destruction in the DMM model of PTOA. Specifically, we found a slight, but significant preference for depth-based damage in the MTP but not the MFC of DMM joints (Figure 1C). This concentrated focal cartilage damage could be the result of mechanical or biological processes that occur preferentially at the medial tibial plateau in response to the induced injury.

Significance: Findings from our combined quantification technique can be utilized to visualize tissue level processes involved in the development/progression of PTOA; these will be followed up by extensive immunohistochemical analysis to study the underlying cellular correlates to these processes. These same procedures will also be repeated in studies utilizing novel therapeutic strategies for preventing PTOA after DMM surgery/knee injury. Furthermore, the expansion of the scoring methods described here could be of benefit to other research groups performing similar structural analyses of cartilage.

References: 1. Gillquist, J., *Sports Med* (1999). 2. Maletius, W., *Am J Sports Med* (1999). 3. Glasson, S.S., *Osteoarthr Cartil* (2010). 4. Glasson, S. S., *Osteoarthr Cartil* (2007). 5. Chambers, M.G., *Arth Rheum* (2001). 6. David, M., *ORS Conference* 2015

2015 ORS

Detailed Quantification of Early Articular Cartilage Structural Changes in the Murine Destabilized Medial Meniscus Model of Post-Traumatic Osteoarthritis

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Disclosures: **M.A. David:** None. **M.K. Smith:** None. **A.T. White:** None. **R.T. Locke:** None. **C. Price:** None.

Introduction: Post-traumatic osteoarthritis (PTOA), an accelerated form of osteoarthritis, is a debilitating disease of articular cartilage caused by traumatic joint injury, e.g. ligament or meniscal tears. PTOA is common in athletes and military service members and up to 50% of those who experience a traumatic joint injury will develop PTOA within 10-15 years [1]. Currently, preventative treatments for PTOA are lacking, potentially due to the fact that preclinical PTOA research has tended to focus on mid-to-end-stage disease, whereas the initial precipitating changes remain largely unknown and untargeted. To improve our understanding of PTOA development we present a preliminary report regarding detailed cartilage structural damage from early through late-stage disease in a murine joint instability model of PTOA, the destabilization of the medial meniscus (DMM) [2]. To determine how and when PTOA-related cartilage structural damage initiates we compared two semi-quantitative scoring paradigms. One system is based on classifying cartilage fibrillation/erosion across the width of articular surface [3] while the other is a modified classification of damage based upon the depth of articular and calcified cartilage erosion/fibrillation in relation to the calcified cartilage/subchondral bone interface [4].

Methods: Surgery and Sample processing: Male C57BL/6 mice (Jackson Labs) underwent DMM surgery at the age of 12-weeks, in which transection of the medial meniscotibial ligament causes medial meniscus extrusion within the joint space resulting in PTOA. Right limbs were utilized for the surgical procedure, and contralateral limbs served as internal controls. Joints were harvested at baseline (0d), or 3-, 7-, 14-, 28-, 56-, 84- and 112-days post-injury. Samples were immediately fixed in 4% paraformaldehyde for 48 hours, decalcified in 14% EDTA for 15 days, embedded in paraffin, and cut in 5 μ m thick coronal sections. To assess PTOA-related structural damage, every 10th section throughout the joint was stained with Safranin-O/Fast Green. From this set five consecutive sections that spanned the majority of the cartilage-cartilage contact surfaces (an $\sim$ 400 μ m wide region) of the tibial plateau and femoral condyle were selected for scoring. Scoring: Three blinded individuals ranked the amount of cartilage damage using either an established semi-quantitative scoring system [3], which included assessing the percentage of cartilage fibrillation/erosion across the width of articular surface (referred to as width-based; Table 1), or a modified version [4] that assessed the percentage of erosion/fibrillation depth in the articular and calcified cartilage with references to the calcified-cartilage/subchondral bone interface (referred to as depth-based; Table 1). Damage was assessed on the medial and lateral femoral condyles and tibial plateaus on a 0-6 scale. Additionally, the degree of synovitis and osteophyte formation were assessed on a 0-3 scale (0=normal, 1=mild, 2=moderate, 3=severe). To establish the natural time-course of PTOA, scoring was performed across all stages of disease development, from 3-days (mild to no damage) to 112-days post-surgery (severe PTOA). For each joint, each scorer's "average", "summed", and "max" score was calculated and then averaged among the three blinded scorers to quantify cartilage damage. By combining both scoring systems we will further construct a

detailed, graded representation of the distribution of both focal and widespread cartilage degeneration from the anterior to posterior regions of the joint at various time points post-injury.

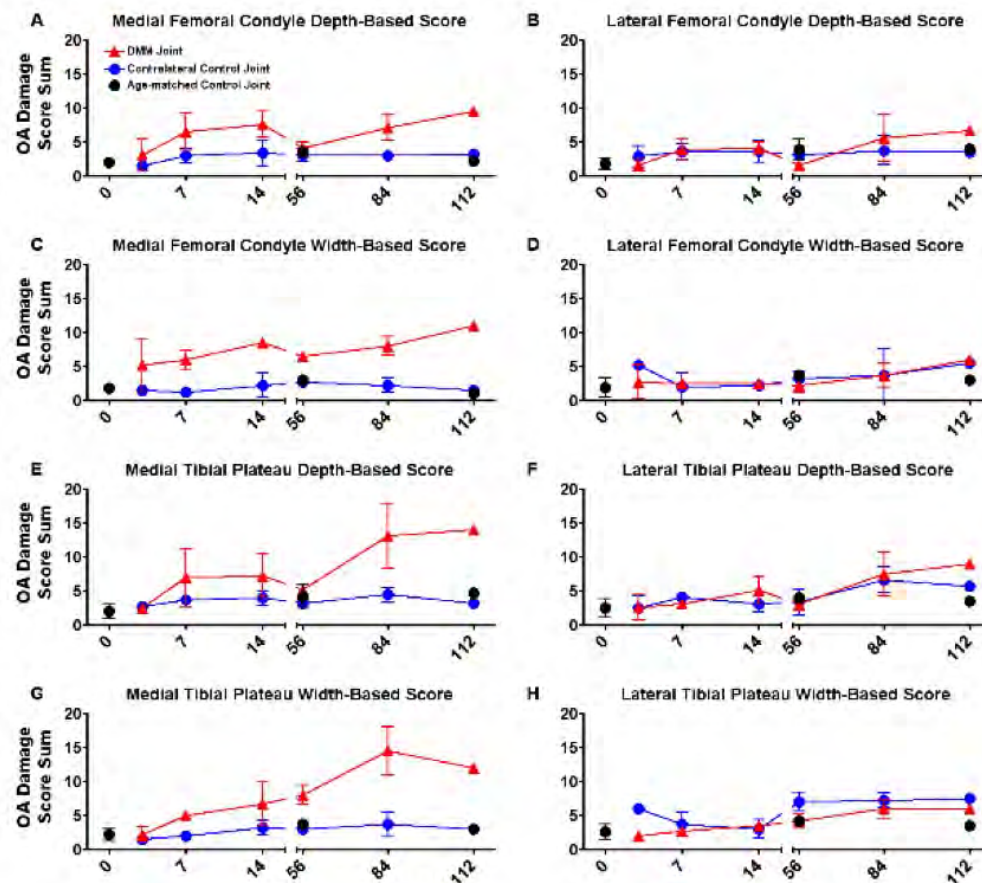
Results: Currently, we only present the results for an $n = 2$ joints for all post-surgery time points, thus only trends regarding the two scoring systems and natural progression of PTOA following DMM can be inferred. Within the cartilage-cartilage contact region of interest of the knee, structural damage was observed to increase with time by both scoring methods in DMM joints, with the most damage seen, as expected, at 112-days post-injury within the medial femoral condyle and medial tibial plateau compartments (Figure 1). Unexpectedly, our data suggested a very-early and rapid increase in cartilage damage scores post-surgery (between 3- and 7-days post-surgery) in the DMM specimens. Interestingly, the lateral tibial plateau (Figure 1F and H) and lateral femoral condyles (Figure 1D) in the contralateral limbs showed increased damage scores at 84-days and beyond compared to age-matched controls, suggesting distant-reaching effects of DMM-injury, which might indicate some degree of biomechanical compensation within the mice. Regarding synovitis and osteophyte formation, there appears to be an early increase in synovitis that gradually decreases overtime while osteophyte formation lags slightly but is quite evident by 7-days post-injury (Figure 2).

Discussion: Combining the two scoring methods in this study allows for a comprehensive view of cartilage fibrillation/erosion width (contact area) and depth scores/distributions within each joint. We provide preliminary results regarding the natural progression of focal and widespread structural damage with murine articular cartilage following DMM-injury. Unexpectedly, an early increase in cartilage damage and osteophyte formation was seen by 7-days post-injury, which suggests cartilage erosion/fibrillation and bone structure responses start earlier than previously appreciated. These results also suggest that studies attempting to prevent PTOA initiation and progression, which this investigation is a prelude to, may require immediate-early interventions to be most efficacious. Ongoing studies utilizing both width- and depth-based scoring systems and micro-CT analysis will permit the specific determination of how PTOA-related cartilage degeneration is initiated and will provide a full view of cartilage damage progression and bone changes over time. Presently, we are continuing to score the remaining joints in our study ($n=5-10$ specimens per time point/grouping). Overall, the results of this study will be used as a baseline to quantify the efficacy of novel, targeted chondroprotective therapeutics to prevent and/or hinder the initiation and progression of PTOA and to relieve the burden of PTOA.

Significance: The quantification of early through late-stage structural damage within articular joints following PTOA-precipitating injury is critical to our understanding of the natural time-course of cartilage degeneration in PTOA, as well as the development of rationally designed therapies for preventing/treating PTOA.

Table 1: Two Scoring Methods for Classifying Damage to Articular Joints Following DMM-Injury

Score	Depth-Based	Width-Based
0	Normal	
0.5	Loss of Safranin-O without structural changes	
1	Roughened surface and small fibrillations without loss of cartilage	
2	Vertical clefts down to the layer immediately below the superficial layer and some loss of surface lamina	
3	Fibrillation/erosion/clefts <25% of articular joint surface depth relative to CC/subchondral bone interface	Fibrillations/erosion to the level of CC, extending <25% of the articular joint surface width
4	Fibrillation/erosion/clefts 25-50% of articular joint surface depth relative to CC/subchondral bone interface	Fibrillations/erosion to the level of CC, extending 25-50% of the articular joint surface width
5	Fibrillation/erosion/clefts 50-75% of articular joint surface depth relative to CC/subchondral bone interface	Fibrillations/erosion to the level of CC, extending 50-75% of the articular joint surface width
6	Fibrillation/erosion/clefts >75% of articular joint surface depth relative to CC/subchondral bone interface	Fibrillations/erosion to the level of CC, extending >75% of the articular joint surface width

**Figure 1:** DMM-related structural damage “summed scores” of joint compartments from both depth-(A,C,E,G) and width-(B,D,F,H)based PTOA scoring systems of DMM, contralateral, and age-matched joints at various time points (0-, 3-, 7-, 14-, 56-, 84- and 112-days) post-surgery (n=2 joints per time-point).

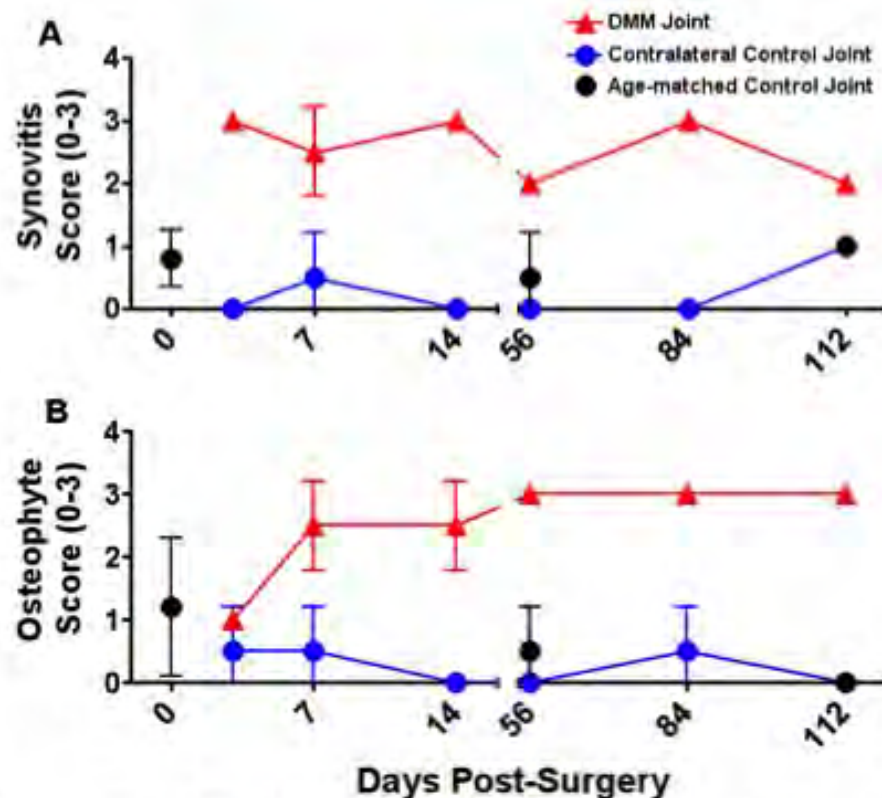


Figure 2: “Max” synovitis (A) and osteophyte (B) scores for DMM, and contralateral and age-matched joints at various time points post-surgery (n=2-joints per time-point).

SPATIO-TEMPORAL QUANTIFICATION OF CARTILAGE STRUCTURAL CHANGES IN A MURINE MODEL OF POST-TRAUMATIC OSTEOARTHRITIS

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INTRODUCTION

Post-traumatic osteoarthritis (PTOA), an accelerated form of OA, results from traumatic joint injury, e.g., ligament and meniscal tears, and is common in active individuals, e.g., athletes and uniformed service members. Approximately 50% of patients that experience a ligamentous tear will exhibit cartilage damage within 10- to 15-years of the injury [1]. Unfortunately, preventative treatments for PTOA are lacking, potentially due to a preclinical focus on mid-to-end-stage disease, whereas the initial changes precipitating PTOA remain largely unknown [2]. In order to develop prophylactic, chondro-protective therapies it is necessary to understand the natural time-course of cartilage structural degradation within the joint during the progression of PTOA. Knowledge of the spatial and temporal progression of cartilage degeneration across the injured joint, heretofore underappreciated, is critical to defining and interpreting the cartilage biology and pathophysiology underlying PTOA, and whether they are of mechanical or cellular origin. Therefore, the objective of the present study was to provide a detailed spatio-temporal histological quantification of cartilage structural changes from early (3-day) through late-stage (112-days) disease in a surgically-induced, murine joint instability model of PTOA, the destabilization of the medial meniscus (DMM) [3]. We hypothesized that cartilage damage would occur early following a traumatic injury and that the cartilage damage would be preferentially localized to the anterior regions of joint where surgical induced joint-instability results in altered biomechanical contact.

METHODS

Animals and Surgeries: Male C57BL/6 mice (Jackson Labs) at the age of 12-weeks underwent DMM surgery in right limbs with contralateral limbs serving as internal controls (n = 5-10mice/group) were harvested at baseline (0d), 3-, 7-, 14-, 28-, 56-, 84- or 112-days

post-injury. Histological Processing: Samples were immediately fixed (in a naturally flexed position), decalcified, embedded in paraffin, and serially cut into 5- μ m thick coronal sections. Starting at the front of the joint (anterior), two-sections were selected approx. every 100- μ m and stained with Safranin-O/Fast Green to assess changes in cartilage structure. From these, five sets of sections spanning the cartilage-cartilage contact (~500- μ m wide) of the tibial plateau and femoral condyle were selected for scoring. Scoring: Scoring was performed across all stages of disease development to establish the natural time-course and spatial distribution of cartilage damage following DMM. Three blinded scorers semi-quantitatively ranked cartilage damage in each section using two separate metrics adapted from prior versions of scoring systems [4-6]. First, the extent of cartilage damage across the width of articular surface (referred to as width-based; Figure 1), and second, the extent of depth-wise articular/calcified cartilage damage (referred to as depth-based; Figure 1). For each metric, cartilage damage was assessed on a 0-6 scale for both the medial and lateral femoral condyles and tibial plateaus, and utilized for both temporal and spatial analysis. For temporal analysis, a single average joint score was aggregated for each specimen based upon each scorer's findings, and used to calculate the group mean and SD for each time point. For spatial analysis, an average score at each section location (anterior to posterior) was aggregated from each scorers findings, and used to calculate a spatial location mean and SD for each location and time point. Statistical Analysis: To establish differences in cartilage damage between DMM and contralateral joints at each time point, paired t-tests well as one-way ANOVA with post-hoc and linear regression across location were performed. Statistical significance was set at $p < 0.05$. Data is presented as mean $\pm$ standard deviation

RESULTS

We found significant, site-specific cartilage damage across the medial compartments of DMM joints. No significant damage was observed in the lateral compartments of DMM joints or in any region of the control joints. Temporally, significant cartilage damage appeared as early as 3-days post-injury in the MTP, observed as a loss of proteoglycan staining (Figure 2). Cartilage structural damage in MTP and MFC continued to increase with time under both scoring methods, with the most damage seen at 112-days post-injury. Spatially, we observed trends of cartilage damage that appeared greater in the anterior region of the tibia compared to posterior (Figure 3); however these findings were only significant at 84- and 112-days post-injury. No differences existed at any time point post-injury within the MFC.

DISCUSSION

By combining two scoring methods with temporal and spatial analyses this study provided a detailed representation of the extent and timing of focal vs. widespread cartilage damage, from anterior to posterior, in the joint following injury. We found early and highly localized cartilage damage within the medial compartments of DMM joints. An initial loss of proteoglycan content (3-d), followed by fibrillations/erosion of articular cartilage (7-d), cumulating in full cartilage loss by 112-days post-injury was observed. When compared to the literature, our observation of cartilage damage from 14-days onward post-DMM is entirely consistent with others findings from DMM models in mouse [7-9], rat [10], and rabbit [11]. However, to the best of our knowledge, no group has investigated cartilage damage from 3-7 days post injury in the murine DMM model of PTOA. Our temporal findings suggest that studies attempting to prophylactically prevent PTOA initiation and progression may require immediate-early interventions (≤ 7 d in mice) to be most efficacious.

Moreover, we also observed a spatial localization of cartilage damage in the MTP, but not in the MFC. While a trend for increased MTP damage anteriorly was seen at several time points, statistical significance was only found at the later time points (84 and 112-days). These findings support the idea that the insult of the DMM causes altered loading and presumably increased localized contact stress at the front of the joint (anteriorly). There was no difference in damage spatially in the MFC, which suggests that the MTP and MFC may experience different mechanical loading and articulation patterns following DMM. Importantly, our spatial findings are consistent with qualitative observations made in the pioneering murine DMM study where increased damage was found anteriorly [3]. In addition, other animal DMM models have shown spatially localized changes in cartilage damage (medial to lateral) [10] as well as increased medial compartment peak contact stresses [11]. Non-invasive murine models have also shown localization of cartilage damage and chondrocyte apoptosis that is dependent on magnitude and location of force applied [12]. Taken together, these studies and ours suggest that insult and altered loading may be an important factor in rapid precipitating and progressing cartilage damage post-injury.

Qualitative analysis of the localization of cartilage damage has additionally suggested that temporal- and spatial-localization of damage to the MTP cartilage, and its subsequent cellular response (loss of chondrocytes), may be related to changes in meniscal coverage of the articular surface following DMM (Figure 4). We are presently quantifying this relationship, as well as investigating if spatial-temporal changes in other joint tissues, e.g., synovium, bone, and menisci, occur post-DMM.

This study is significant in that it's the first to provide a detailed analysis of the spatio-temporal cartilage changes within the murine DMM model of PTOA from early (3-day) through late (112-day) stage

disease. Overall, our findings establish a baseline for the detailed study of the cellular and molecular pathobiology of PTOA, as well as its prevention through the use of novel, targeted, prophylactic chondro-protective therapeutics.

ACKNOWLEDGEMENTS

This research was funded by DoD PR120788P1. Special thanks to Brianna Gietter and Fiona Flowerhill for their assistance in this project.

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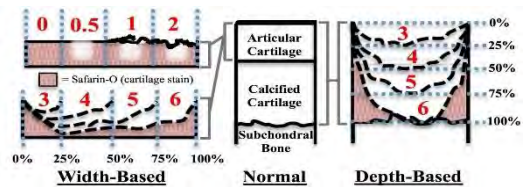


Figure 1: Schematic of width- and depth-based scoring on 0-6 scale (0 normal and 6 being the most cartilage damage). Scoring of 0-2 in both systems are identical. Width is scored irrespective of depth, and vice versa.

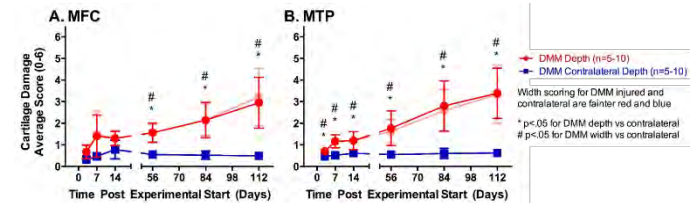


Figure 2: Temporal quantification of cartilage damage width- and depth-wise in the MFC (A) and MTP (B) post-DMM.

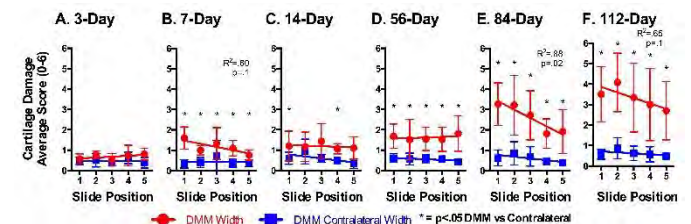


Figure 3: Spatial quantification of cartilage damage, width-wise in MTP post-DMM (A-F) from anterior (position 1) to posterior (position 5) regions of the joint. Regression data only indicated for DMM.

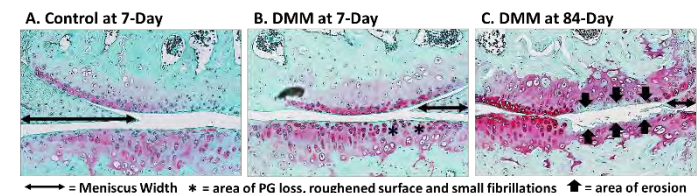


Figure 4: Extrusion of meniscus with medially localized cartilage damage in control (A) & DMM joints at 7- & 84-d (B&C) [position 2 shown].

Title: Repeated Intra-Articular Injection of Zoledronic Acid Suppresses Cartilage Erosions After DMM**Authors:** Michael David¹, Melanie Smith¹, Rachael Pilachowski¹, John Lowman¹, Christopher Price^{1,2}¹Biomedical Engineering, ²Mechanical Engineering, University of Delaware

Introduction: Post-traumatic osteoarthritis (PTOA), accelerated cartilage degeneration due to traumatic joint injury, is incurable and a significant socioeconomic burden. In searching for PTOA modifying therapeutics, the use of bisphosphonates (BPs), including the potent 3rd generation BPs (e.g. zoledronic acid [ZA]) have been explored. Systemically delivered, high-dose ZA has demonstrable pre-clinical anti-osteoarthritic and anti-inflammatory efficacy¹⁻⁴. Nonetheless, the significant skeletal side-effects of continuous, high-dose, systemic ZA treatment⁵ represents a barrier to clinical acceptance. However, recent work has shown that ZA has direct, beneficial effects on chondrocyte metabolism and health *in situ*⁶; and macrophage depleting activities, which can reduce inflammation in a variety of diseases⁷. These results suggest that local delivery of BPs, via intra-articular (i.a.) injection, represents an alternative strategy for using ZA in preventing PTOA; yet, the study of ZA in this manner is nascent. We hypothesize that *i.a. injection of ZA, through its direct chondroprotective and anti-inflammatory activities, will suppress cartilage damage and degenerative joint changes post-injury*. The objective of this study was to establish the efficacy of locally injected ZA into the knee as an anti-osteoarthritic and anti-inflammatory therapeutic in a mouse model of PTOA, the destabilization of the medial meniscus (DMM).

Materials and Methods: *Surgery and Sample Processing (IACUC approval received):* Adult (12wk old), male C57BL/6 mice underwent DMM surgery⁸ in their right limbs and were randomly separated into 4 groups: DMM only, DMM + immediate (d0) i.a. ZA, DMM + delayed (7d) i.a. ZA, and DMM + repeated (0, 7, 14, 21d) i.a. ZA. Joints were harvested at 7-, 14-, 56-, and 84-days post-DMM (n=5-10 mice/group), processed for paraffin embedding and microtomy. Coronal sections (~100- μ m apart) spanning the mid-joint contact region were stained with Safranin-O, fast green, and hematoxylin, and semi-quantitatively scored⁸. *Histological Scoring:* 3 blinded individuals evaluated the severity of medial joint cartilage damage (0-6 scale), and the degree of synovitis and osteophyte formation/maturation (0-3 scale each)⁹. *Statistics:* unpaired t-tests were used to determine differences between DMM and ZA treated groups at each time point; statistical significance was set at $p < 0.05$.

Results and Discussion: In terms of synovitis, single and repeated i.a. ZA injections failed to modify synovitis post-DMM (Figure 1A&B; data only shown for multiple injection). Single i.a. ZA injection (0d & 7d) resulted in a transient delay in osteophyte maturation early on (14-days), however, osteophyte maturation was no from DMM controls at later time points. In contrast, repeated i.a. ZA injection delayed osteophyte maturation throughout the study (Fig. 1A&C). Most importantly, single i.a. ZA injection had no effect on cartilage damage compared to DMM alone. In contrast, multiple i.a. ZA injections suppressed cartilage erosion at later time points (84-days) compared to DMM (Fig. 2A&C). Although these findings hold initial promise for the clinical translation of i.a. ZA in treating PTOA, optimization of overall delivery strategy for i.a. ZA injection is required: including i) timing of delivery, ii) ZA concentration, and iii) optimization of delivery platform, as ZA is rapidly cleared from the joint following i.a. injection. Furthermore, since inflammation is typically a source of pain in PTOA, alternative delivery strategies that improve anti-inflammatory efficacy should be identified.

Conclusions: In this study, we demonstrated that *repeated, i.a. ZA can suppress joint changes and cartilage erosions, but not inflammation, post-DMM*, establishing a framework to investigate the *in vivo* mechanisms underlying i.a. ZA treatment of PTOA, and for exploring its translation as an alternative PTOA therapeutic.

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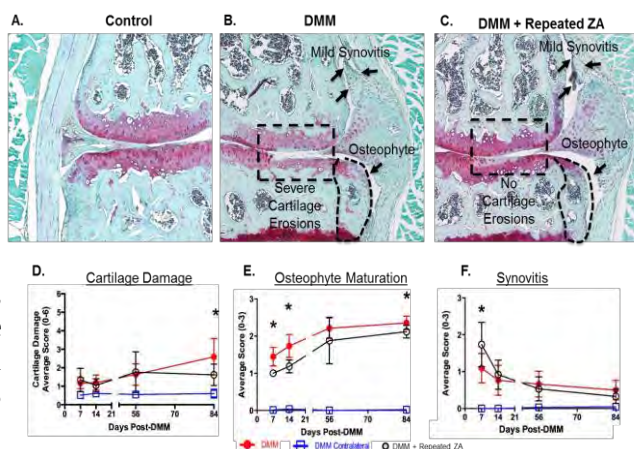


Figure 1. Representative 84-day post-DMM histology highlighting the suppression of cartilage erosions and osteophyte maturation, but not synovitis, with repeated i.a. ZA (C) compared to DMM (B); no degenerative joint changes are seen in control joints (A). Semi-quantitative scoring of cartilage damage (D.), osteophyte maturation (E.), and synovitis (F.) confirm the qualitative efficacy of repeated ZA as anti-osteoarthritic.

Destabilization of the Medial Meniscus in Mice Induces Rapid and Focal Progression of Chondrocyte Proliferation and Death

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Introduction: Chondrocytes residing within the articular cartilage (AC) are critical in maintaining cartilage homeostasis and integrity. Chondrocyte apoptosis is a clinical hallmark of osteoarthritis (OA) and is also observed in models of post-traumatic osteoarthritis (PTOA) [1-3]. In PTOA it is suspected that chondrocyte death is related to alterations in joint loading resulting from traumatic joint injury. For example, in the murine destabilization of the medial meniscus (DMM) model of PTOA, chondrocytes death has been observed as early as 1-week post-DMM [4,5]. Our ongoing work using this model has identified an immediate loss of chondrocytes in regions of the AC that experienced a loss medial meniscus coverage due to DMM-induced meniscal extrusion (manuscript in submission). However, the cause of this loss of chondrocytes remains unknown. Additionally, in an overload-induced ACL rupture model of PTOA, chondrocyte apoptosis has been shown to localize to regions of altered mechanical loading [6]. Furthermore, it has been suggested that these changes may be reflective of injured chondrocytes undergoing processes similar to endochondral ossification, leading to cartilage degradation through the activation of chondrocytes proliferation followed by hypertrophy and apoptosis [7]. However, a complete spatiotemporal analysis of chondrocyte death and proliferation in the murine DMM model of PTOA and whether these changes are related to cartilage structural damage has yet to be established. Therefore, we utilized a set of spatiotemporal evaluation methods to characterize patterns of chondrocyte proliferation and apoptosis from early (3-day) through late (112-days) timepoints post-DMM. We hypothesized that early progressive changes in chondrocyte proliferation followed by apoptosis would be localized to AC regions that experience a loss of meniscal coverage due to DMM-induced meniscal extrusion.

Methods: Surgery and Sample Processing: Right limbs of 12-week old C57BL/6 male mice (Jackson Labs) underwent DMM-surgery [8] with contralateral limbs serving as internal controls. At 3-, 7-, 14-, 56-, 84- and 112-days post-DMM (n = 5-10 per time point) knee joints were harvested, processed for paraffin embedding, and cut into 5-µm thick coronal sections. Three sections spanning ~300-µm of the cartilage-on-cartilage contact were stained histologically and immunohistochemically to assess the progression of chondrocyte death and proliferation in the medial compartment tibial plateau. To determine chondrocyte viability, sections were stained with hematoxylin and eosin (H&E; hematoxylin for cell chromatin/DNA), DAPI (intact cell DNA), and TUNEL assay (apoptosis). Chondrocyte proliferation was determined immunohistochemically via Ki-67. **Data analysis:** To quantitate chondrocyte changes, we used a custom, semi-automatic MATLAB algorithm to manually count positive cells for each stain in the medial tibia plateau (MTP) AC. The number of positive cells were automatically determined across four quadrants of the MTP AC width (quad 1 [inner most region of joint] through 4 [closest to joint margin]). A representative value for each quadrant was derived by averaging all three sections. **Statistics:** To determine differences between DMM and contralateral limbs, we performed paired t-test. Statistical significance was set at p<.05.

Results: We identified distinct spatiotemporal patterns in chondrocyte loss, apoptosis, and proliferation in the MTP AC from DMM joints, supporting our hypothesis. Regarding cell viability, the presence of chondrocytes containing intact DNA/chromatin (hematoxylin-positive in H&E) was reduced in quad 3 as early as 3-days, and continued to decrease post-DMM (Fig. 1C and Fig. 2A). With time, chondrocyte loss spread to quadrants adjacent to quad 3, with quad 2 and 4 experiencing a significant loss of chondrocytes only at later timepoints (56-days and beyond; Fig. 1B and D) while quad 1 did not experience loss of chondrocytes (Fig. 1A). Early loss of chondrocytes co-localized to quadrants exhibiting TUNEL positive staining as early as 3-days. These regions also appear to co-localize with regions exhibiting a loss of DAPI-stained chondrocytes (Fig. 2B). By 7- and 14-day, chondrocyte apoptosis was observed to have spread to quad 2 and 4 in the AC. In terms of chondrocyte proliferation, we observed no appreciative pattern for Ki-67 in any quadrant at 3-days, although positive cells were observed (Fig. 2C). However, by 7- and 14-days, proliferation was distinctly observed in quadrant 2.

Discussion: In this study, we observed the appearance of a distinct spatiotemporal progression of chondrocyte proliferation and apoptosis following DMM. An immediate (within 3-days) reduction in chondrocyte number was seen by H&E and DAPI in regions where the AC experienced acute altered femur-on-meniscus-on-tibia contact following injury (Q3). In these regions TUNEL staining co-localized with areas lacking DAPI-positive cells, consistent with these cells undergoing apoptosis and mirroring findings from a murine PTOA model of overload-induced ACL rupture [6]. While our study does not shed light onto the specific processes occurring prior to 3-days post-injury we did note that from 3-days onward a spatiotemporal progression of proliferation and apoptosis was observed. Tissue regions initially exhibiting increased Ki67 expression at early timepoints subsequently exhibited increased TUNEL positive staining and loss of chondrocytes, resulting in lockstep spread of chondrocyte proliferation and apoptosis and cell loss to adjacent AC quadrants. These findings indicate a temporal pattern of chondrocyte proliferation followed by apoptosis in response to injury. Quantitative analysis of these observations is currently ongoing, as well as studies investigating whether these chondrocytes are undergoing cellular processes similar to endochondral ossification and how these processes influence cartilage degeneration following joint injury. However, we note that chondrocyte proliferation/clustering has been observed in early OA, [7,9], suggesting that proliferation may be a common chondrocyte response to altered joint environments. Based upon these findings we suggest that chondrocyte proliferation may be a key, albeit detrimental, injury-response mechanism. We posit that altered loading patterns can activate chondrocyte proliferation and endochondral progression. However, the production of matrix degrading enzymes and paracrine factors required to support these processes may further accelerate the degradation of an AC extracellular matrix that is already under aberrant mechanical loading, contributing to the vicious cycle of cartilage degeneration. Future studies are warranted to define the relationship between chondrocyte proliferation, apoptosis, and catabolic processes post-injury.

Significance: This study has identified a localized population of chondrocytes that undergo a distant spatiotemporal pattern of proliferation and apoptosis in the articular cartilage following the destabilization of the medial meniscus in the mouse. This knowledge is critical to better understanding the cellular and molecular pathoetiology of PTOA and for the testing of therapeutics to prevent PTOA-related cartilage cellular changes in this pre-clinical model.

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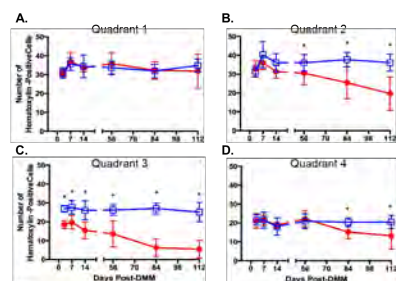


Figure 1. Spatial Quantification of Chondrocyte Presence Following DMM. The number of hematoxylin-positive chondrocytes in quadrants 1 through 4 (A, through D.) of the medial tibial plateau. Data shown as mean $\pm$ STD (n = 5/time point/group). * = p<0.05 for paired t-test between DMM (red) and contralateral joints (blue). Similar patterns were observed for the number of DAPI-positive cells.

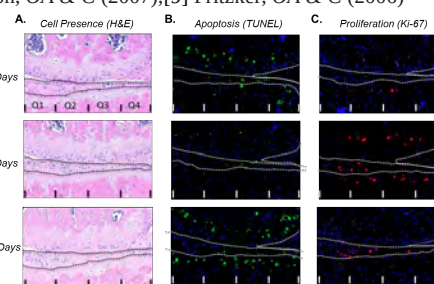


Figure 2. Spatiotemporal patterns of chondrocyte loss, apoptosis, and proliferation. A loss of chondrocytes was observed in the AC (A.; black dotted line) In addition, chondrocytes were observed to undergo apoptosis (B.; green) and proliferation (C.; red) from 3 through 14 days in specific quadrants (Q1-4) of the articular cartilage (dotted white line). Cells with intact cell DNA are shown in blue.

Whole-Joint Structural Consequences Following Destabilization of the Medial Meniscus in the Mouse

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Disclosures: No disclosure

Introduction: Post-traumatic osteoarthritis (PTOA), the accelerated degeneration of cartilage due to a traumatic joint injury, is now appreciated as a whole joint disease. Thus, tissues extending beyond articular cartilage, such as subchondral bone, synovium, menisci, and osteophytes, may contribute to the initiation and progression of cartilage damage following joint trauma. In the destabilization of the medial meniscus (DMM) model of PTOA, the synovium becomes inflamed (increased cellularity and thickness), osteophytes form at the joint margin, and the subchondral bone may become sclerotic [1-3]. However, the complete spatiotemporal evolution of such tissue-specific changes post-injury and the relationship of these changes with cartilage damage remains unknown. Defining these spatiotemporal changes may provide knowledge towards understanding which tissues contribute to injury-induced progression of cartilage degradation. Therefore, we employed histological scoring and micro-CT (μ -CT) analysis to characterize structural changes in the synovium, osteophytes, menisci, and underlying bone from early (3-day) through late-stage (112-days) disease in the murine DMM model of PTOA.

Methods: Surgery and Sample Processing: Right limbs of 12-weeks old male C57BL/6 mice were subjected to either DMM- or sham-surgery (DMM, n=5-10 mice/group and Sham n=3 mice/group, respectively) [4]. For both DMM and Sham, contralateral limbs served as internal controls. Joints were harvested at 3-, 7-, 14-, 28-, 56-, 84- and 112-days post-DMM. For histological analysis, samples were fixed, decalcified, embedded in paraffin, and cut (5- μ m thick coronal sections). Every 20th section (~100 μ m apart) was stained with Safranin-O, fast green, and Weigert's iron hematoxylin. From these, five sections spanning the joint's mid-contact cartilage region (~500 μ m) were selected for semi-quantitative scoring and labeled (1-5), from the anterior to posterior. **Histological Scoring:** Three blinded individuals evaluated the degree of synovitis and osteophyte formation/maturation in the medial compartments using semi-quantitative histological scoring metrics on a 0-3 scale. For synovitis 0=normal, 1=mild, 2=moderate, and 3=severe and for osteophytes 0=none, 1=cartilaginous template, 2=cartilaginous template/bone, 3= fully developed bone [5]. For each level, a representative value was derived from all scorers and used for whole-joint (averaged among all levels) and spatial analysis (per level). **μ -CT analysis:** For micro-CT (μ -CT) analysis, DMM, Sham, and contralateral joints were scanned using a μ -CT scanner (6 μ m/voxel; Scanco uCT-35). To quantitate structural changes in calcified tissues, the calcified meniscus, intracapsular ectopic bone, subchondral bone, and epiphyseal bone were manually segmented from 3D images and processed using CTan software. **Statistics:** To determine differences between surgical and contralateral limbs, we performed paired t-tests. To identify spatial differences in joint damage, linear regression between damage score and joint position (anterior-to-posterior) was performed. Statistical significance was set at $p < .05$.

Results: As reported previously, we identified the general appearance of both synovitis and developing osteophytes as early as 3-days in the medial compartment of DMM joints (data not shown) [5]. With time, the degree of synovitis in DMM joints lessened in severity but still persisted, while in Sham joints, synovitis was observed only at 7-days likely due to the surgical insult. In DMM joints, osteophytes were observed to mature from a cartilaginous template into bone by 56-days, while osteophytes were not observed in Sham joints. Spatially within DMM joints, we observed a preferential increase in synovitis anteriorly compared to posteriorly at all timepoints, however no spatial preferences in osteophyte presence and maturation was observed (Fig.1). Regarding morphological changes in the calcified tissues, we observed that both the total and mineralized volume of the medial meniscus, which is calcified in the mouse, increased at late timepoints within DMM joints (Fig. 2), with the greatest changes appearing anteriorly. In addition, we observed the significant formation of ectopic intracapsular bone at 56-days and beyond. DMM joints exhibited no significant changes in subchondral bone or epiphyseal bone structure (data not shown).

Discussion: In this study, we identified an immediate (3-day), focal (anterior-medial) and persistent development of synovial inflammation and hyperplasia (synovitis) post-DMM. These spatiotemporal findings are consistent with previous work in the DMM model suggesting that inflammation may aid in the degeneration of cartilage [1]. Consistent with previous studies [2], the development of osteophytes was similarly rapid with an extensive elaboration of an endochondral templates in the medial joint compartment (3-day), followed by progressive calcification, and bone and hyaline cartilage formation. Future studies are warranted to determine if the degree of synovitis and osteophytes prior to 3-days and whether these tissues influence each other's progression. Similar to previous studies, we did not observe any significant changes in the structure of medial or lateral subchondral or epiphyseal bone compartments by μ -CT following DMM. However, we did observe previously unreported/unacknowledged changes in the morphology and volume of the medial meniscus and the accumulation of heterotopic bone formation at later time points (56-days and beyond). This could suggest that increased calcification of these tissues may represent an injury-driven response to restabilize the joint. While this study provides new knowledge of whole joint changes following DMM-injury, the future study of how they contribute to the progression of cartilage damage is warranted and may provide novel targets for both therapy and treatment.

Significance: This study provides a spatiotemporal characterization into the development of synovitis, osteophytes, and ectopic bone in the murine DMM model of PTOA and establishes a baseline to study the cellular and molecular changes regulating these tissue responses. These findings may shed insight towards identifying targets and treatments for mitigating these tissue changes and their possible role in cartilage degeneration following joint trauma.

Acknowledgements: This study was supported by a grant from the US DOD CDMRP PRMRP PR120788P1.

References: [1] Jackson, *Arthritis Rheumatol* (2014); [2] Loeser, *PLoS ONE* (2013); [3] Iijima, *Osteoarthritis and Cartilage* (2014); [4] Glasson, *Osteoarthritis and Cartilage* (2007); [5] David, *ORS Annual Meeting* (2015).

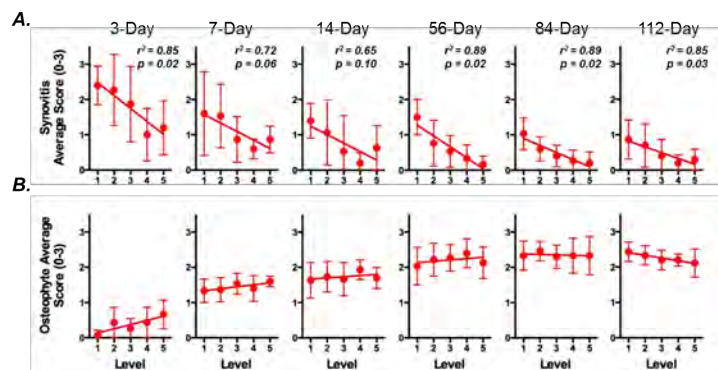


Figure 1. Anterior-to-Posterior Distribution of Synovitis and Osteophyte Formation/Maturation Post-DMM. Semi-quantitative scores of synovitis (A.) and osteophyte formation (B.) in medial compartments of DMM joints. Data shown as mean $\pm$ STD (n = 5-10/time point/group).

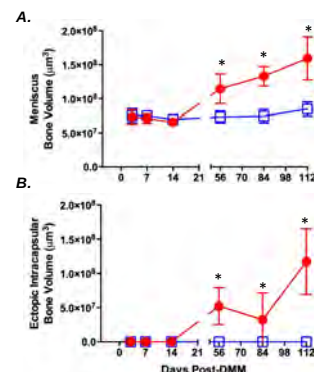


Figure 2. μ CT Quantification of Bone and Tissue Volume in the Meniscus and Ectopic Intracapsular Post-DMM. Bone volume (A.) from the medial meniscus and ectopic intracapsular bone (B.) of DMM (red circles) and DMM contralateral (blue square). Data shown as mean $\pm$ STD (n = 5/time point/group). * = $p < .05$ t-test between DMM and contralateral

Repeated Intra-Articular Injection of Zoledronic Acid Suppresses Cartilage Erosions Following Destabilization of the Medial Meniscus in Mice

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Introduction: Post-traumatic osteoarthritis (PTOA), an accelerated form of cartilage damage resulting from traumatic joint injury, is incurable and poses a significant socioeconomic burden. In the search for PTOA modifying therapeutics, recent studies have used bisphosphonates (BPs), including the newer and more potent 3rd generation nitrogen containing BPs (nBPs; e.g. zoledronic acid [ZA]). Systemically delivered, high-dose ZA has demonstrated anti-osteoarthritic efficacy in animal models [1-3], including the murine destabilization of the medial meniscus (DMM) model of PTOA [4]. Nonetheless, the risk of significant skeletal side-effects due to high-dose nBP treatment [6] represents a barrier to the clinical acceptance of systemically delivered nBPs for preventing PTOA. However, recent work has shown that ZA can have direct, beneficial effects on the metabolism and health of chondrocytes in situ [7]. Furthermore, ZA has macrophage depleting activities [8] and can reduce macrophage-mediated inflammation in a variety of diseases [9,10]. These results suggest that local targeting BPs to the joint, e.g. via direct intra-articular (i.a.) injection, may represent an alternative strategy for utilizing nBPs to prevent PTOA; yet, the application of nBPs in this manner has been nascent. We hypothesized that i.a. injection of nBPs, through their direct chondroprotective and anti-inflammatory activities, will suppress cartilage damage and degenerative joint changes following joint injury. Therefore, the objective of this study was to determine the efficacy of i.a. injection of the nBP ZA as an anti-osteoarthritic therapeutic in the murine DMM model of PTOA.

Methods: Surgery and Sample Processing: Adult, male C57BL/6 mice (Jackson Labs) underwent DMM-surgery [11] in their right limbs at 12-weeks of age and were randomly separated into four groups: DMM with no treatment (DMM), DMM + an immediate single i.a. ZA injection, DMM + a single 1wk post-DMM i.a. ZA injection, DMM + i.a. ZA injections at 0-d, & 1-, 2-, and 3-wk post-DMM. i.a. ZA injections were administered at a dose of .64 ng/kg per injection (ZA monohydrate) based upon previous *in situ* work conducted by our collaborator [7]. Joints were harvested at 7-, 14-, 56-, and 84-days post-DMM (n=5-10 mice/group), processed for paraffin embedding, and cut into 5-µm thick coronal sections. Sections ~100-µm apart were stained with Safranin-O, fast green, and Weigert's iron hematoxylin. From these, five sections spanning the joint's mid-contact cartilage regions were selected for semi-quantitative scoring. **Histological Scoring:** By using semi-quantitative histological scoring systems, three blinded individuals evaluated the severity of width-wise cartilage damage on a 0-6 scale [12], and the degree of synovitis and osteophyte formation/maturation on a 0-3 scale [12]. Whole-joint damage, synovitis, and osteophyte scores were then derived for each specimen (averaged all sections). **Statistics:** To determine differences in cartilage damage, synovitis, and osteophyte scores between DMM and ZA treated groups at each time point, unpaired t-tests were performed. Statistical significance was set at p<.05.

Results: Intra-articular injection of either single or repeated doses of ZA generated differential results with regards to their influence on synovitis, osteophyte maturation, and cartilage degeneration following DMM. In terms of synovitis, both single and repeated i.a. ZA injections failed to reduce the degree of synovial inflammation post-DMM (Figure 1A&B; data only shown for multiple injection). Regarding osteophyte formation, single i.a. ZA injections (0d and 7d) resulted in a transient delay in osteophyte maturation early (14-days), however, at later time points osteophyte formation was no different between these groups and DMM controls (data not shown). In contrast, repeated i.a. ZA injections tended to delay osteophyte maturation (seen as delay in the transition from a cartilaginous tissue to calcified bone) throughout the study (Figure 1A&C). Most importantly, single i.a. ZA injections had no effect on cartilage damage in the medial femoral condyle (MFC) or medial tibial plateau (MTP) compared to DMM alone (data not shown). In contrast, multiple i.a. ZA injections did not appear to alter the early progression of superficial cartilage damage (14d; Fig 2B), but did suppress cartilage erosion at the later time point (84-days) compared to DMM alone (Figure 2A&C).

Discussion: In this study, we demonstrated the ability of intra-articular injections of the nBP ZA to suppress joint changes and cartilage degradation associated with PTOA in the murine DMM model. We found that only repeated i.a. ZA injections provided a significant degree of long-term anti-osteoarthritic efficacy, through its ability to delay the maturation of osteophytes at the joint margins and to suppress cartilage erosions. Unfortunately, i.a. injection of ZA did not appear to have any anti-inflammatory effects as hypothesized, at least using the present histological scoring techniques. Although these findings hold initial promise for the clinical translation of i.a. ZA in treating PTOA, optimization of overall delivery strategy for i.a. ZA injection is required. For instance, the optimal: **i)** timing of delivery, **ii)** concentration of ZA, and **iii)** delivery platform needs to be determined, as ZA is rapidly cleared from the joint following i.a. injection (data not shown). Furthermore, since inflammation is typically a source of pain in PTOA, we hope that alternative delivery strategies that also provide a degree of anti-inflammatory efficacy can be identified and leveraged. Lastly, the cellular and molecular mechanisms underlying the beneficial results observed with repeated i.a. ZA injections *in vivo* have yet to be identified, and form the basis of continuing investigations.

Significance: This study identified that repeated intra-articular administration of the nBP ZA can provide a degree of anti-osteoarthritic efficacy following joint injury in a murine model of post-traumatic osteoarthritis (DMM). This research establishes a framework to investigate the *in vivo* mechanisms underlying the efficacy of i.a. ZA in treating PTOA, and for exploring the translation of i.a. ZA to the treatment of PTOA following joint injury.

Acknowledgements: This research was supported by a grant from the US DOD CDMRP PRMRP PR120788P1

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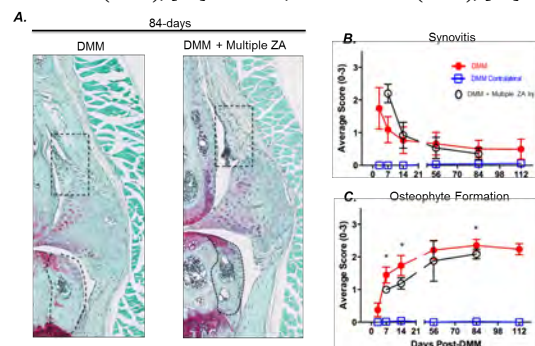


Figure 1. Effect of Repeated I.A. ZA Injection on Synovitis and Osteophyte Formation Post-DMM. Representative Safranin-O stained sections identifying synovitis (dotted black box) and osteophyte formations (black trace) from DMM and DMM + Repeated ZA injections at 84-days. Scoring of whole-joint synovitis (B.) and osteophyte formation (C.) in medial compartments following DMM-surgery. Data shown as mean $\pm$ STD (n = 5-10/time point/group). * = p<0.05 for unpaired t-test between DMM (red) and DMM + ZA joints (black).

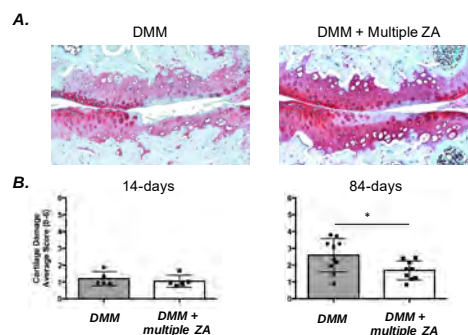


Figure 2. Effect of Repeated I.A. ZA Injection on Cartilage Damage Post-DMM. Representative Safranin-O stained sections from a DMM and DMM + multiple ZA joint at 84-days (A.). Scoring of medial compartment cartilage damage at 14- and 84-days following DMM (B.). Data shown as mean $\pm$ STD (n = 5-10/time point/group). * = p<0.05 for unpaired t-test between DMM and DMM + multiple ZA joints.

Destabilization of the Medical Meniscus in Mice Induces Early, Focal Changes in Cartilage Cellularity and Structure

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Introduction: Post-traumatic osteoarthritis (PTOA) is an accelerated form of cartilage damage due to traumatic joint injury. Such injuries can lead to destabilization of the joint and consequentially, altered joint loading. To recapitulate changes in joint loading and their role in PTOA progression, the murine destabilization of the medial meniscus (DMM) model of PTOA has been used extensively. Using this model, our laboratory and others have identified a loss of cartilage structural integrity as early as 3-days following DMM that increases in severity with time [1-3]. In addition, chondrocyte death, which may play a large role in initiation and progressing cartilage damage, has been observed in this model throughout disease progression [1-2]. Collectively, these studies have established a time-frame for the initiation and progression of cartilage damage and chondrocyte death post-DMM. However, investigation into the spatial aspects and correlates of these temporal changes is lacking. For example, our qualitative impression of the DMM literature has suggested a heretofore overlooked co-localization of cartilage damage to regions of the articular surface experiencing acute and persistent changes in their coverage by the medial meniscus (herein referred to as meniscal coverage) as a result of DMM-induced meniscal extrusion. Defining the anterior-to-posterior and medial-to-lateral cellular and structural changes and their relationships to meniscal extrusion may provide insight into the mechanisms by which PTOA initiates and progresses. Therefore, we utilized a set of spatiotemporal scoring and evaluation methods to characterize the spatiotemporal changes in cartilage cellularity and structure and their relationship with meniscal extrusion and cartilage degeneration from early (3-day) through late-stage (112-days) disease in the murine DMM model of PTOA. We hypothesized that DMM-induced meniscal extrusion leads to rapid and focal changes in cartilage cellularity and structure in specific regions of the joint that experience altered femur-on-meniscus-on-tibia contact following joint destabilization.

Methods: Surgery and Sample Processing: Male C57BL/6 mice underwent DMM-surgery (n=5-10 mice/group) [4] in the right limbs; contralateral limbs served as internal controls. Joints were harvested at 3-, 7-, 14-, 56-, 84- and 112-days post-DMM and fixed in 4% paraformaldehyde at a standard flexion angle of ~80°, processed for paraffin embedding, and cut in 5-µm thick coronal sections. Every 10th slide (~100 µm apart) was stained with Safranin-O, fast green, and Weigert's iron hematoxylin. A subset of five slides spanning the joint's cartilage-on-cartilage contact region was selected for semi-quantitative scoring and immunohistochemical analysis. The slides were labeled (1-5), from the anterior to posterior levels of the joint. **Histological Scoring:** Three blinded individuals evaluated the extent of width-wise cartilage damage in the medial compartments using a semi-quantitative histological scoring system on a 0-6 scale [3] and both a whole-joint (averaged among all levels) and spatial (per level) damage scores were collated. **Quantification of Meniscal Coverage and Chondrocyte Presence:** To determine the spatial (medial-to-lateral) and temporal relationships between meniscal extrusion/coverage and chondrocyte cellularity and cartilage damage, we stained sections (immediately adjacent to the Safranin-O section) for type II collagen and DAPI-positive cells. Using a custom, semi-automated MATLAB algorithm, we traced the medial tibial plateau articular cartilage (AC), calcified cartilage (CC), and meniscus and counted the number of DAPI-positive cells in AC and CC (Fig. 2A). From these traces and counts, we determined the degree of meniscal coverage (length of meniscus/width of articular surface) and number of DAPI-positive cells in four quadrants (quad 1 [inner most region of joint] through 4 [closest to joint margin]) of the AC and CC. **Statistics:** To determine differences between DMM and contralateral joints, spatial distributions anteriorly to posteriorly, and relationships between cellular changes and meniscal coverage, we performed paired t-tests, linear regression, and correlation analysis, respectively. Statistical significance was set at p<.05 and all analyses were performed in Prism 6.

Results: We found immediate and focal changes in cartilage structure and cellularity in the medial compartments of the DMM joints. Temporally, we previously reported that cartilage experienced a significant loss of safranin-O staining and minor surface fibrillations as early as 3-days post-DMM, which developed into full AC erosions by 84-days [3]. This cartilage damage was preferentially localized anteriorly in the joint at late time points (Fig. 1), consistent with the location of the anterior-transection of the medial meniscal tibial ligament. Cartilage damage also appeared to co-localize to the regions (quad 3) of the articular cartilage surface that experienced a marked reduction in meniscal coverage as early as 3-days (Fig. 2A and B). More importantly, the number of DAPI-positive cells was found to be significantly reduced in quad 3 of DMM joints (Fig. 2C; data not shown for other quadrants). Furthermore, the number of DAPI-positive cells in quad 3 linearly correlated with meniscal coverage (Fig. 2D), suggesting a relationship between focal loss of cellularity and meniscal extrusion. No significant differences in the number of DAPI-positive cells were observed in the CC (data not shown).

Discussion: In this study, we provide a complete semi-quantitative and quantitative analysis of cartilage cellularity and structural changes from early through late timepoints in the murine DMM model of PTOA. Our findings of anteriorly localized damage are consistent with original qualitative observations and altered joint mechanics following DMM-surgery [5]. Furthermore, we identified the rapid and focal accumulation of cartilage damage and chondrocyte loss to specific regions of AC that experienced a loss of meniscal coverage. This confirms our original qualitative impressions and suggests that a loss/change of meniscal coverage may initiate mechanical or biological processes leading to cartilage damage. Importantly, the loss of chondrocytes coinciding in regions where long-term cartilage erosions also develop (Q3) suggests that this focal population of chondrocytes may be critical in the initiation of long-term cartilage degeneration. However, the extent of chondrocyte loss occurring immediately following DMM (0-3 days) as well as the cellular mechanisms regulating this chondrocyte loss remain unknown and warrant future investigations.

Significance: This study provides evidence for a role of DMM-mediated meniscal extrusion in the induction of rapid and focal cartilage cellular and structural changes in the anterior-to-medial regions of the joint. Furthermore, this study has identified a focal population of chondrocytes that can be targeted to study the cellular and molecular pathoetiology of PTOA, and for developing and evaluating chondroprotective treatments and strategies.

Acknowledgements: A grant from the US DOD CDMRP PRMRP PR120788P1 supported this research.

References: [1] Loeser, *PLoS ONE* (2013); [2] Leahy, *Arthritis Rheum* (2015); [3] David, *ORS Annual Meeting* (2015); [4] Glasson, *Osteoarthritis and Cartilage* (2007); [5] Moodie, *Osteoarthritis and Cartilage* (2011)

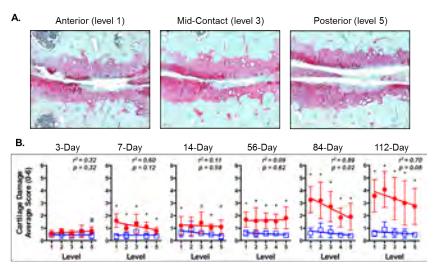


Figure 1. Spatial distribution of cartilage damage within the medial compartment of DMM joints. Representative safranin-O stained sections of medial compartment cartilage damage from the anterior-to-posterior of a 84-day post-DMM joint (A) and the spatiotemporal scoring of damage. Results shown as mean ± STD (n = 5/time point/group); *p<.05 and #p<.10 (trend) for paired t-test between DMM and contralateral joints at indicated level. Linear regression data are shown for DMM joints only.

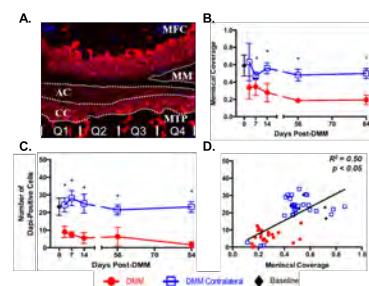


Figure 2. Medial-to-lateral distribution of meniscal coverage and cartilage cellularity post-DMM. Representative section for type II collagen (red) and DAPI-positive cells (blue)(A). Meniscal coverage (B) and number of DAPI-positive cells in quadrant 3 following DMM (C). Correlation between DAPI-positive cells and meniscal coverage in Q3 (D). Results shown as mean ± STD (n = 5/time point/group); *p<.05 for paired t-test between DMM and contralateral joints