AWARD NUMBER: W81XWH-19-1-0335
Log Number: PR180268P1

TITLE: Proteomics of Knee Osteoarthritis

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CONTRACTING ORGANIZATION: University of California, Davis

REPORT DATE: October 2021

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
October 2021	Annual	9/1/2020 – 8/31/2021

4. TITLE AND SUBTITLE
Proteomics of Knee Osteoarthritis

5a. CONTRACT NUMBER
W81XWH-19-1-0335

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
MRL/MpJ (super healers) mice have a unique ability to repair wounds and are protected from cartilage degradation subsequent to joint trauma. The hypothesis is that in response to injury, MRL/MpJ mice synthesize proteins that (1) protect the joint from cartilage degradation and/or (2) promote cartilage regeneration. The PIs propose to generate an atlas of the injury-activated proteome in mouse models with varying susceptibility to posttraumatic osteoarthritis (PTOA): (1) C57BL/6; (2) C57BL/6 treated with streptozotocin (STZ), a model of type 1 diabetes; (3) MRL/MpJ (super healers); and (4) STR/ort (spontaneous OA). By conducting comparative proteomics of injured and uninjured joints, the PIs will identify novel protein candidates for further exploration as potential therapies for treating injured joints. The project's specific aims are (1) application of in vivo metabolic labeling to quantify and characterize de novo protein synthesis, cellular proliferation, and mineral apposition in injured joints of mice with varying susceptibility to PTOA and (2) identification of newly synthesized RNA and proteins in the articular cartilage and immune cells of injured knees using a liquid sample interface for the AMS instrument in combination with liquid chromatography-mass spectrometry (LC-MS).

15. SUBJECT TERMS

NONE LISTED

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	21	**19b. TELEPHONE NUMBER** *(include area code)*

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INTRODUCTION:

MRL/MpJ (super healers) mice have a unique ability to repair wounds and are protected from cartilage degradation subsequent to joint trauma. The hypothesis is that in response to injury, MRL/MpJ mice synthesize proteins that (1) protect the joint from cartilage degradation and/or (2) promote cartilage regeneration. The PIs propose to generate an atlas of the injury-activated proteome in mouse models with varying susceptibility to posttraumatic osteoarthritis (PTOA): (1) C57BL/6; (2) C57BL/6 treated with streptozotocin (STZ), a model of type 1 diabetes; (3) MRL/MpJ (super healers); and (4) STR/ort (spontaneous OA). By conducting comparative proteomics of injured and uninjured joints, the PIs will identify novel protein candidates for further exploration as potential therapies for treating injured joints. The project's specific aims are (1) application of in vivo metabolic labeling to quantify and characterize de novo protein synthesis, cellular proliferation, and mineral apposition in injured joints of mice with varying susceptibility to PTOA and (2) identification of newly synthesized RNA and proteins in the articular cartilage and immune cells of injured knees using a liquid sample interface for the AMS instrument in combination with liquid chromatography-mass spectrometry (LC-MS).

KEYWORDS:

MRL/MpJ; STR/ort; osteoarthritis, post-traumatic osteoarthritis, diabetes, streptozotocin, MetRS, superhealer, chondrocytes, knee joint, anterior cruciate ligament, ACL, de novo protein synthesis, PTOA

ACCOMPLISHMENTS

For the 2nd year of this grant, our main focus has been on conducting tasks associated with Aim 1/Major Task 1 (Sub Aim 1A) of the proposal, following the original tasks and timeline we are highlighting in 'green' subtasks that have been competed, in 'yellow' subtasks that have started and are in progress, and in 'blue' subtasks that have not yet started but will initiate in the next funding period.

Specific Aim 1.	Timeline (months)	Status	Site 1 (LLNL)	Site 2 (UCD)
Major Task 1. (Sub Aim 1A): Quantify *de novo* protein synthesis in injured joints	**1-24**			
Subtask 1.1. Obtain IACUC/ACURO approval; breed MRL/MpJ, Str/Ort and C57BL/6 cohorts.	1-12	completed	Breed animals	
Subtask 1.2. Induce traumatic OA in 10 week old MRL/MpJ, ~~STR/ort~~ C3H/HeJ mice. Mice with receive AHA/^{14}C-threonine; 336 mice will be used (2 genotypes x 24 mice per group x 7 time points).	3-15 3-36	In progress	Prepare cohorts of animals, transport to UCD for injury	336 animals will be injured, return animals to LLNL post injury
Subtask 1.3. Sample collection from 336 animals from Subtask 1.2	3-15 3-36	In progress	Dissect joints, extract proteins	microCT (168 scans)
Subtask 1.4. Induce type 1 diabetes in 6 week old C57BL/6 mice for 4 weeks.	7-9	completed	Administer STZ	
Subtask 1.5. Induce traumatic OA in 10 week old C57BL/6 and STZ mice. Mice with receive AHA/^{14}C-threonine; 336 mice will be used (2 treatments x 24 mice per group x 7 time points).	10-18	completed	Prepare cohorts of animals, transport to UCD for injury	336 animals will be injured, return animals to LLNL post injury
Subtask 1.6. Sample collection from 336 animals from Subtask 1.5	10-18	completed	Dissect joints, extract proteins	microCT (168 scans)
Subtask 1.7. AMS analysis to quantify	10-24	In progress	^{14}C measurements	

^{14}C-threonine levels in injured and uninjured animals	10-36		by AMS	
Subtask 1.8. BONCAT analysis	10-24 10-36	In progress	Click-chemistry; quantification	
Subtask 1.9. FUNCAT/Histological analysis	10-24 10-36	In progress	Embed, section, visualize proteins	Embed, section, visualize proteins
Milestone 1: IACUC/ACURO Approvals				
Milestone 2: Complete Sample Collection for STR and MRL strain				
Milestone 3: Complete Sample Collection for diabetic mice				
Milestone 4: Complete Proteomic Analysis for 1, 3, 5 and 7 days post injury				
Milestone 5: Complete Proteomic Analysis for 14, 21 and 42 days post injury				
Milestone 6.1: Manuscript #1 describing injury-induced phenotypic and molecular changed in T1D mice				
Milestone 6.2: Manuscript #2 describing injury-induced proteomic changed in mouse strains with varying susceptibility to PTOA				

What was accomplished under these goals?

Subtask 1.1. Obtain IACUC/ACURO approval; breed MRL/MpJ, Str/Ort and C57BL/6 cohorts.

Completed in year 1.

Subtask 1.2. Induce traumatic OA in 10 week old MRL/MpJ, STR/ort mice. Mice with receive AHA/^{14}C-threonine; 336 mice will be used (2 genotypes x 24 mice per group x 7 time points).

For this task, we have focused primarily on comparing C57BL/6 to MRL/MpJ. Unfortunately, we have had breeding difficulties with the STR/ort line (as mentioned in year 1 annual report) and at this point this strain is no longer breeding, so the samples collected thus far will be insufficient to make robust conclusions. We have replaced this strain with the C3H/HeJ (C3H) strain, since work conducted in prior work by a former graduate student in the lab, Ms. Melanie Mendez has identified the C3H strain as having susceptibility to PTOA. In the next funding period we will begin the characterization of this strain and compare it to C57BL/6.

Subtask 1.3. Sample collection from 336 animals from Subtask 1.2

Injuries were resumed on 9/15/2020, we are continuing to collect samples, stockpile them. Because of social distancing restriction where only 2 scientists were allowed to work together in the same space, these large animal experiments have been slow, and we have had to break them down in smaller experiments to ensure collection is feasible and does not compromise the quality of the protein extractions. Therefore, we do not yet have full sets with all the controls and the required biological replicates to ensure rigor and statistical significance. We require 5 animals per timepoint, per genotype, per injury, per treatment (PBS vs AHA), therefore for 1 experiment, per strain we need 20 animals per gender per timepoint. The proteomic analysis will initiate in the next fiscal year when we will have completed the collection for full sets. We anticipate day 7 and 14 post injury to be the 1st datasets to initiate the proteomic analysis in year 3.

Subtask 1.4. Induce type 1 diabetes (T1D) 6 week old C57BL/6 mice for 4 weeks.

Completed in year 1.

Subtask 1.5/1.6. Induce traumatic OA in 10 week old C57BL/6 and STZ mice.

Completed in year 1.

In this subtask we 1st determined whether type 1 diabetes mellitus (T1DM) is a risk factor for primary osteoarthritis (OA) by comparing the knee joints of control and T1DM mice at 16-weeks of age **(Figure 1A)**. Non-fasting blood glucose levels were significantly elevated ($p<0.005$) at 10 and 16-weeks of age in the T1DM group, whereas control mice maintained lower blood glucose levels <300 mg/dL. Body weight was also assessed throughout the experiment; STZ treated mice had lower body weight and maintained similar levels throughout the experiment when compared to the control mice ($p <0.0001$). OA development was evaluated using Safranin-O and Fast Green staining. Control mice exhibit healthy cartilage characterized by a smooth and strong Safranin-O staining and no bone degradation was visible on either the femoral condyle or tibia **(Figure 1B)**. In sharp contrast, T1DM mice exhibited loss of Safranin-O staining and minor fibrillation, suggesting the loss of proteoglycan content **(Figure 1B)**. The articular cartilage layer also appeared slightly thinner in some regions in the T1DM joints than in the controls **(Figure 1B, yellow arrows)**. OA severity was quantified using a modified Osteoarthritis Research Society International (OARSI) grading scale and the OA score was found to be significantly higher ($p <0.05$) in the T1DM group. These results confirm that T1DM is a risk factor for primary OA, and STZ-induced T1DM mice display mild OA phenotypes at 16-weeks of age **(Figure 1B)**.

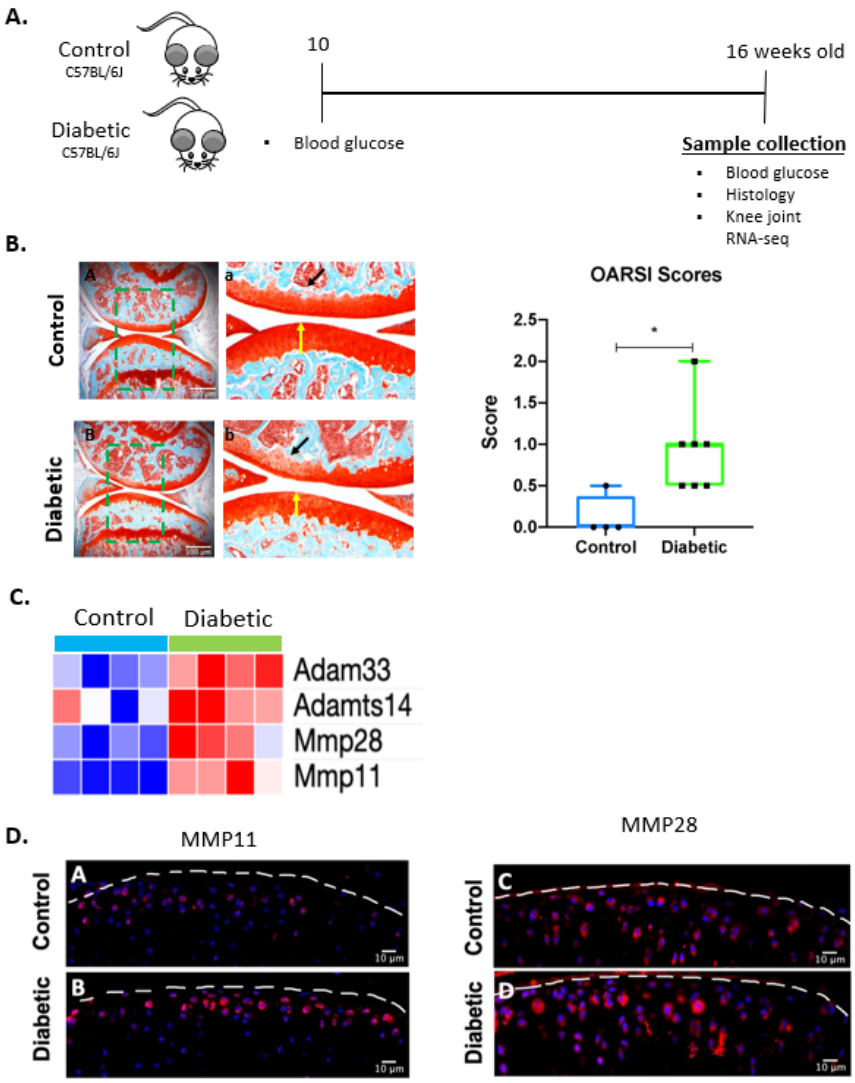


Figure 1. T1DM promotes mild OA in C57BL/6J male mice. (A) Schematic representation of the experimental design. (B) Histological evaluation of the knee joint of 16 weeks old control and T1DM mice using Safranin-O (red: cartilage) and Fast Green (green: surrounding tissue) (5x magnification; scale bars: 100um). High magnification images corresponding to green boxes (A, B) are provided a,b. OA severity was quantified using the Osteoarthritis Research Society International (OARSI) scoring system. (C) Heat map of selected matrix degrading enzymes. (D) Protein expression of MMP11 and MMP28. Blue: DAPI staining showing the nucleus, Red: staining showing the protein of interest (20x magnification; scale bar 10um). Bar graphs values are average ± standard error; control (c) n=5, diabetic (D) n=10 per group. Statistical analysis performed by t-test.

To understand how T1DM promotes primary OA, at the molecular level, we also compared whole knee joint RNA-sequences (RNA-seq) between 16-weeks old control and T1DM mice. Computational analyses identified 519 up- and 645 down- regulated genes differentially expressed in T1DM joints relative to control joints. Among the 519 up-regulated genes in T1DM joints, we identified several genes encoding for matrix degrading enzymes including matrix metallopeptidase 11 (*Mmp11*) and (*Mmp 28*), metalloproteinase 33 (*Adam 33*) and metallopeptidase 14 (*Adamts 14*) **(Figure 1C)**. Consistent with the gene expression data, immunohistochemistry staining confirmed higher protein levels of MMP11 and MMP28 in the articular cartilage of T1DM mice compared to controls **(Figure 1D)**, suggesting that these enzymes may be responsible for the emerging OA phenotype observed in the T1DM group. Other significantly up-regulated genes of interest included extracellular matrix and skeletal development associated genes. Diabetes is also well known to decrease bone mineral density and we found elevated expression levels of several bone remodeling markers in the T1DM group including *Bglap*, *Acp5*, *Cstk*, *Oscar* and *Pth1r*. Significantly down-regulated genes were enriched in

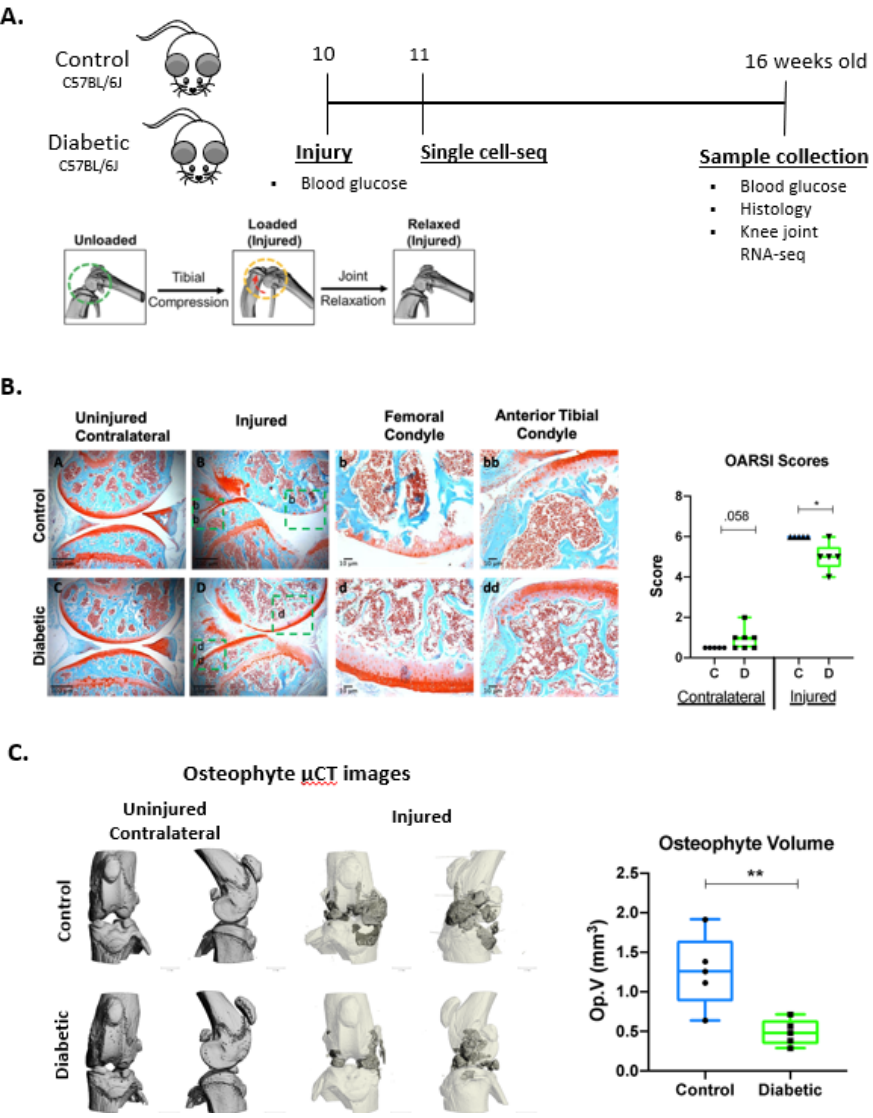


Figure 2. Pre-existing T1DM prevents PTOA in C57BL/6J male mice. (A) Schematic representation of the experimental design. (B) Histological evaluation of uninjured and injured control and diabetic mice at 6 weeks post-injury using Safranin-O (red: cartilage) and Fast Green (green: surrounding tissue) (5 and 20x magnification; scale bars: 100 and 10um). PTOA severity was quantified using the OARSI scoring system. (C) Osteophyte imaging using µCT and osteophyte volume at six weeks post-injury. Bar graphs values are average ± standard error; n=5, per group. Statistical analysis performed by t-test.

several functional categories, including markers of *bone mineral content* and *skeletal muscle tissue development and morphology*.

Next, we injured 10-weeks old T1DM and C57Bl6 control mice **(Figure 2A)**. Six-weeks post injury, joints were harvested and PTOA phenotypes were examined by histology and microcomputed tomography (µCT). Consistent with previous results, injured control mice displayed severe cartilage degradation with significant loss of Safranin-O staining and most of the femoral head was lacking the articular cartilage layer **(Figure 2B)**. In sharp contrast, injured T1DM mice more closely resembled the uninjured T1DM controls. Cartilage thickness was preserved, and while some proteoglycan staining loss was observed in the deep layer, the superficial layer was unchanged, suggesting a significantly slower progression to PTOA in the T1DM mice **(Figure 2B)**. Quantification of OA severity using the OARSI grading scale system showed significantly higher cartilage score in the injured control group than in the injured T1DM group **(Figure 2B**; $p<0.05$**)**. Subchondral trabecular bone mass and osteophyte volume was also quantified by µCT. Consistent with prior reports that T1DM promotes bone loss, uninjured T1DM mice had significantly less trabecular bone volume fraction (BV/TV) and less trabecular thickness compared to the control mice. While joint injury also significantly affected bone mass in the control mice, trabecular bone mass was not affected in the T1DM group the same as in the controls. No additional significant bone loss was observed in the subchondral bone of injured T1DM mice when compared to uninjured T1DM or injured control mice. Another characteristic of PTOA is the formation of osteophytes. T1DM mice were also protected from osteophyte formation in response to injury, where significantly less ectopic bone was quantified around the injured joints of the T1DM group **(Figure 2C)**. These results suggest a slower progression of PTOA in T1DM mice, this result was exciting but contrary to our original hypothesis. This data has been drafted into a manuscript.

Subtask 1.7. AMS analysis to quantify 14C-threonine levels in injured and uninjured animals

This work has not initiated, samples are still being collected

Subtask 1.8. BONCAT analysis

This work has not initiated, samples are still being collected

Subtask 1.9. FUNCAT/Histological analysis

Histological analyses have been completed for T1DM, C57BL6 and MRL

In year 3 we will replace STR with a different strain, these samples will be collected and analyzed in year 3.

Major Tasks 2 work will initiate in the next fiscal year. STR/Ort strain will be replaced with C3H/HeJ.

Major Task 2. (Sub Aim 1B) Quantify cell proliferation in injured joints	**13-27**		
Subtask 2.1. Breed necessary cohorts of MRL/MpJ, ~~STR/Ort~~ C3H/HeJ and C57BL/6.	13-18 **24-36**	Breed animals	
Subtask 2.2 Induce traumatic OA in 10 week old MRL/MpJ, ~~STR/ort~~ C3H/HeJ, STZ and C57BL/6 mice. Mice with receive ^{14}C-thymidine; 336 mice used (4 genotypes x 12 mice per group x 7 time points).	16-24 **24-36**	Prepare cohorts of animals, transport to UCD for injury	336 animals will be injured, return animals to LLNL post injury
Subtask 2.3. Sample collection from 336 animals from Subtask 2.2	16-24 **24-36**	Dissect joints, extract DNA	
Subtask 2.4. AMS analysis to quantify ^{14}C-thymidine levels	19-27 **24-36**	^{14}C measurements by AMS	
Milestone 7: Complete Sample Collection for MRL, STR, STZ and B6 mouse strain			
Milestone 8: Complete AMS analysis			
Major Task 3. (Sub Aim 1C): Quantify mineral apposition in injured joints:	**19-33**		
Subtask 3.1. Breed necessary cohorts of MRL/MpJ, ~~STR/Ort~~ C3H/HeJ, and C57BL/6 cohorts.	19-24	Breed animals	
Subtask 3.2 Induce traumatic OA in 10 week old MRL/MpJ, STR/ort mice, STZ and C57BL/6 mice. Mice with receive ^{45}Calcium. 192 mice will be used (4 genotypes x 12 mice per group x 4 time points).	22-30	Prepare cohorts of animals, transport to UCD for injury	192 animals will be injured, return animals to LLNL post injury
Subtask 3.3. Sample collection from 192 animals from Subtask 3.2.	22-30	Dissect joints, extract DNA	microCT (192 scans)
Subtask 3.4. LC analysis to quantify ^{45}Calcium-levels in injured and uninjured animals	25-33	Measure ^{45}Ca by liquid scintillation	
Milestone 9: Complete Sample Collection for MRL, STR, STZ and BL6 mouse strain			
Milestone 10: Complete microCT/LC analysis			
Milestone 11: Manuscript #2 describing injury induced cellular proliferation and osteophyte formation in mouse strains with varying susceptibility to PTOA.			

Specific Aim 2 and Major Task 4 work was supposed to initiate in the next fiscal year, however, we have already initiated some work on these tasks. One change to the original plan that is worth mentioning, is that we have had increasing difficulties breeding the STR/Ort strain of mice. These mice are giving us consistent small litters, of 1-2 pups and it has been unsustainable to have breeders and generate cohorts for experiments. As an alternative to this OA susceptible strain, we are therefore searching for alternative strains of mice that produce a spontaneous OA phenotype, in particular we are considering using the C3H/HeJ mouse strain in the future.

Specific Aim 2.	Timeline	Status	Site 1 (LLNL)	Site 2 (UCD)

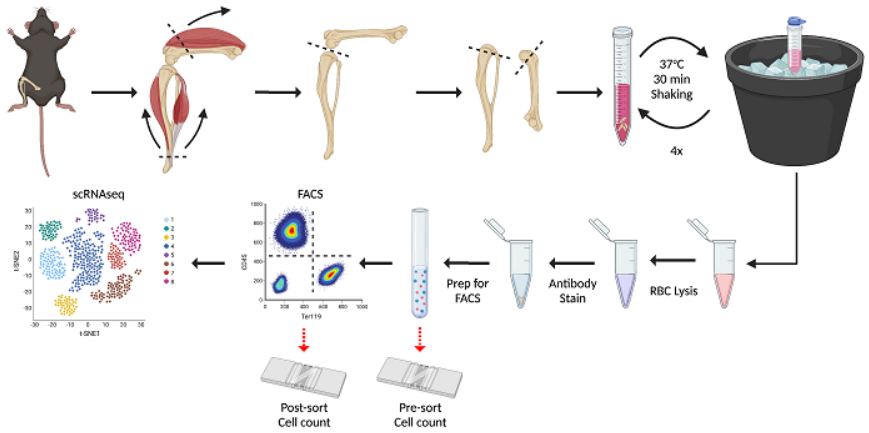
Major Task 4. (Sub Aim 2A): Characterize the injury-induced transcriptome and proteome in the articular cartilage.	**10-33**			
Subtask 4.1. Breed necessary cohorts of ***Ai9; Col2-ER-Cre; MetRS*** animals.	10-21	in progress	Genotype and breed animals	
Subtask 4.2 Induce traumatic OA in 10 week old ***Ai9; Col2-ER-Cre; MetRS*** mice. Mice with receive ^{14}C-threoninine and ANL. 432 mice will be used (2 genotypes x 24 mice per group x 9 time points).	16-24	In progress	Prepare cohorts of animals, transport to UCD for injury	432 animals will be injured, return animals to LLNL post injury
Subtask 4.2A. Conduct scRNA-seq on articular chondrocytes of uninjured and injured C57Bl/6.	12-24	Completed	Prepare cohorts of animals, transport to UCD for injury, isolate single cell, conduct RNA-seq and computational analyses	Injure animals, return animals to LLNL post injury
Subtask 4.2B. Conduct scRNA-seq on immune cells of uninjured and injured C57Bl/6.	12-24	Completed	Prepare cohorts of animals, transport to UCD for injury, isolate single cell, conduct RNA-seq and computational analyses	Injure animals, return animals to LLNL post injury
Subtask 4.3. Sample collection from 432 animals from Subtask 4.2.	16-24	In progress	extract proteins, isolate RNA	
Subtask 4.4. AMS analysis to quantify ^{14}C-threonine levels in injured and uninjured animals	19-30	not started yet	^{14}C measurements by AMS	
Subtask 4.5. BONCAT analysis	19-30	not started yet	Click-chemistry; quantification	
Subtask 4.6. FUNCAT/Histological analysis	19-30	not started yet	Embed, section, visualize proteins	Embed, section, visualize proteins
Subtask 4.7. LC-MS/MS analysis	22-33	not started yet	Protein identification	
Milestone 12: Complete Sample Collection for *Col2-ER-Cre; ~~UPRT;~~ MetRS*				
Milestone 13: Complete Chondrocyte-Specific RNA-seq analysis (scRNA-seq now)				
Manuscript published:				
Sebastian A, McCool JL, Hum NR, Murugesh DK, Wilson SP, Christiansen BA and Loots GG. Single-Cell RNA-Seq Reveals Transcriptomic Heterogeneity and Post-Traumatic Osteoarthritis-Associated Early Molecular Changes in Mouse Articular Chondrocytes. ***Cells*** 2021 June 10(6):1462. DOI: 10.3390/cells10061462.				

Book chapter accepted: McCool JL, Hum NR, Sebastian A, Loots GG. Isolation of Murine Articular Chondrocytes for Single Cell RNA or Bulk RNA Sequencing Analysis. *Methods in Molecular Biology* book entitled "Cartilage Tissue Engineering" Editors Prof. Martin Stoddart, Dr. Angela Armiento, Dr. Elena Della Bella. Springer. To be published in 2022.				
Milestone 14: Complete Chondrocyte-Specific Proteomic Analysis				
Milestone 15: Manuscript #3 describing injury-mediated chondrocyte specific protein and gene expression				
Major Task 5. (Sub Aim 2B): Characterize the injury-induced transcriptome and proteome in the immune system.	**13-36**			
Subtask 5.1. Breed necessary cohorts of ***Ai9; Csf1r-Cre; ~~UPRT;~~ MetRS*** animals. These mice will now be ***Csf1r; Ai9; MetRS***	13-25	In progress	Genotype and breed animals	
Subtask 5.2 Induce traumatic OA in 10 week old ***Ai9; Csf1r-Cre; ~~UPRT;~~ MetRS*** mice. Mice will receive 4TU, ^{14}C-threoninine and ANL. 432 mice (2 genotypes x 24 mice per group x 9 time points). This aim has been modified—UPRT/4TU will not be used and the RNA-seq task will be replaced with single cell sequencing	19-27	In progress	Prepare cohorts of animals, transport to UCD for injury	432 animals will be injured, return animals to LLNL post injury
Subtask 5.3. Sample collection from 432 animals from Subtask 4.2. ScRNA-seq has been conducted on uninjured joints	19-27	In progress	Extract proteins, isolate RNA	
Subtask 5.4. Breed MRL/MpJ animals.	19-25	In progress	Breeding	
Subtask 5.5. Injure MRL/MpJ animals, administer AHA+^{14}C-threonine. 104 mice (12 mice x 9 time points)	19-25	In Progress	Transport cohorts to UCD for injury	104 animals will be injured, return to LLNL
Subtask 5.6. Sort macrophages and T-cells from joints, isolate proteins	21-27	not started yet	FACs, protein extraction	
Subtask 5.7. AMS analysis to quantify ^{14}C-threonine levels in injured and uninjured animals	22-33	not started yet	^{14}C measurements by AMS	
Subtask 5.8. BONCAT analysis	22-33	not started yet	Click-chemistry; quantification	
Subtask 5.9. FUNCAT/Histological analysis	22-33	not started yet	Embed, section, visualize proteins	Embed, section, visualize proteins
Subtask 5.10. LC-MS/MS analysis	25-36	not started yet	Protein identification	
Subtask 5.11. IHC validation	31-36	not started yet	Visualization of	

			C57Bl/6 and MRL/MpJ protein expression	

Milestone 16: Complete Sample Collection for *Ai9; Csf1r-Cre; ~~UPRT;~~ MetRS*
Milestone 17: Complete Immune-Specific RNA-seq analysis (scRNA-seq now)
Milestone 18: Complete Immune-Specific Proteomic Analysis
Milestone 19: Manuscript #4 describing injury-mediated immune specific protein and gene expression, highlighting MRL/MpJ specific proteins that may contribute to PTOA resistance

For Major Task 4. (Sub Aim 2A): Characterize the injury-induced transcriptome and proteome in the articular cartilage. We originally proposed to carry out cell type specific RNA-seq analysis using the UPRT allele bred with the MetRS transgene to generate the ***Ai9; Csf1r-Cre; UPRT; MetRS***. However, the advent of single cell-sequencing technology has proved to a more reliable and informative approach to generating cell-type specific RNA-seq data. We therefore shifted our focus on scRNA-seq and optimized isolation of chondrocytes from mouse joints.



Uninjured C57B6L/6J (B6) and MRL/MpJ (MRL) joints were euthanized at 10-weeks (10W) of age (n=5), and both hindlimbs were collected by removing the leg at the hip joint. The synovial capsule and long bones were cleaned of muscle, tendon and ligaments. The cleaned long bones and joints were then separated from each other in an enzymatic cocktail to also account for cells within the synovial fluid of the joint, then digested using Collagenase 2, to a single cell suspension. Flow cytometry was completed for isolation of chondrocytes by negatively selecting for CD45 (immune) and Ter119 (erythroid) cells. Libraries were prepared for the double negative population using Chromium Single Cell 3′ GEM, Library & Gel Bead Kit v3 following manufacturers protocol and then sequenced using Illumina NextSeq 500. Following sequencing, data was demultiplexed, quality checked, and aligned to the mouse genome (mm10) using Cell Ranger (10X Genomics, CA, USA). Data analysis was completed using Seurat R package and Loupe Cell Browser (10X Genomics, CA, USA). This methodology is outlined in Figure 3 and was drafted as a methods book chapter in *Methods in Molecular Biology* book entitled "Cartilage Tissue Engineering'.

Figure 3. Flowchart of chondrocyte isolation for scRNA-seq application.

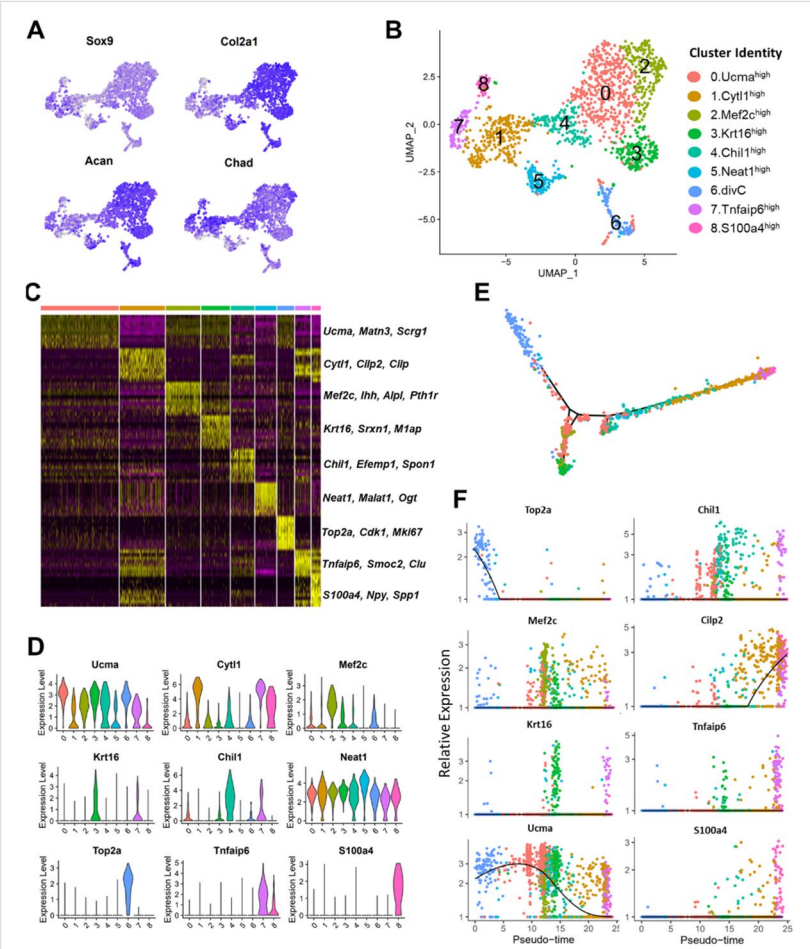


Figure 4. **Characterization of chondrocyte subtypes.** (**A**) Feature plots showing the expression of key chondrocyte markers. (**B**) UMAP plots of various chondrocyte subtypes in mouse knee joints. Colors indicate clusters of various cell types with distinct gene expression profiles. (**C**) Heatmap showing the scaled expression of top genes differentially expressed in each cluster. (**D**) Violin plots showing the expression of selected markers of various chondrocyte subtypes. (**E**) Monocle pseudotime trajectory colored based on chondrocyte clusters in (**A**). (**F**) Expression of chondrocyte subtype markers on a pseudotime scale (colored based on clusters in (**A**)).

The mammalian joint is a complex tissue with multiple cell types including chondrocytes, mesenchymal stem cells (MSCs), osteoblasts, fibroblasts and immune cells, and gene expression profiles derived from whole joint RNA prevents us from distinguishing specific cell type contributions to maintenance of cartilage homeostasis and response to injury. Single-cell RNA sequencing (scRNA-seq) is a new technology that allows us to profile gene expression in individual cells in a tissue with complex architecture, providing a high-resolution view of cellular differences and a better understanding of the function of a given cell. Analysis of connective tissue-forming cells from the joint of 10W old B6 mice identified 10 cell types/subtypes including multiple chondrocyte and fibroblast subpopulations with distinct gene expression profiles. All cells expressing chondrocyte markers (Fig. 4A) were extracted and re-examined in greater detail. This included 1625 cells expressing high levels of *Sox9*, *Col2a1*, and *Acan* (Fig. 4A), accounting for ~65% of all stromal cells analyzed. The analysis of these chondrocytes revealed 9 clusters with distinct gene expression profiles (Fig. 3B–D). Cluster 0 showed enrichment for *Ucma*, *Matn3*, *Papss2*, and *Scrg1* (Fig. 4C,D) and was annotated 'Ucma^{high}'. These genes were also enriched in clusters 2, 3, and 6, but these clusters had additional cluster-specific markers. Cluster 2 showed enrichment for *Mef2c*, *Ihh*, and *Pth1r*, markers of pre-hypertrophic chondrocytes, and this cluster was named 'Mef2c^{high}' (Fig. 3C,D). A subset of cell from the Mef2c^{high} cluster also expressed *Col10a1*, a marker of chondrocyte hypertrophy. Cluster 3 had unique markers *Krt16*, *M1ap*, *Ngf*, and *Srxn1* and was annotated as 'Krt16^{high}' (Fig. 3C,D). Cluster 6 expressed high levels of cell cycle-associated genes *Cdk1*, *Top2a*, *Cenpf*, and *H2afz*, and was identified as 'dividing chondrocytes' (divC).

Cluster 1 expressed high levels of cytokine-like 1 (*Cytl1*), *Cilp2*, and *Prg4*, and was annotated 'Cytl1^{high}' (Fig. 4C,D). Clusters 7 and 8 also robustly expressed these genes, but cluster 7 showed enrichment for additional genes including *Tnfaip6*, *Smoc2, Clu, and Gas1*, whereas cluster 8 showed enrichment for fibroblast/fibrosis markers, fibroblast-specific protein 1 (S100a4/FSP1), and *Col1a1, Col3a1*, and *Abi3bp*, in addition to *Npy* and *osteopontin* (OPN)/secreted phosphoprotein 1 (*Spp1*) (Fig. 4C,D). Cluster 7 was annotated as 'Tnfaip6^{high}' and cluster 8 was annotated as 'S100a4^{high}'. Tnfaip6^{high} cluster also expressed many fibroblast markers and regulators of fibrosis including *Abi3bp, Inhba*, and *Spp1*, but the expression levels for many of these genes were not as high as in the S100a4^{high} cluster. It is likely that the Tnfaip6^{high} cluster represents pre-fibrotic chondrocytes and the S100a4^{high} cluster represents chondrocytes with a more mature fibrotic phenotype. Cluster 4 expressed high levels of *Chil1* (CHI3L1), *Efemp1, Spon1, Mgp*, and *Fxyd3*, and was annotated 'Chil1^{high}' (Fig. 4C,D). Cluster 5 shared several markers with other subtypes but also had higher expression of *Neat1*, *Malat1, Ogt,* and *Wwp2*, and was denoted the 'Neat1^{high}' cluster. To understand the relationship among these chondrocyte subpopulations, we constructed a transcriptional trajectory of these cells on a pseudotime scale

using Monocle. Pseudotemporal trajectory analysis predicted a branched trajectory where divCs resided at one end, while Tnfaip6^{high} and S100a4^{high} cells were observed at the opposing end of the trajectory (Fig. 4E,F). Cytl1^{high} cells were closer to the Tnfaip6^{high} and S100a4^{high} clusters. Cells from the Chil1^{high} and Neat1^{high} clusters resided along the trajectory (Fig. 4D,E), while the Krt16^{high}, and Mef2c^{high} clusters formed distinct branches (Fig. 4E). Ucma^{high} cells resided closer to cells from the Krt16^{high} and Mef2c^{high} clusters and DivCs. This analysis suggested that the Tnfaip6^{high}, S100a4^{high}, and Cytl1^{high} clusters were closer in developmental time while Krt16^{high} and Mef2c^{high} clusters were developmentally closer to the Ucma^{high} cluster, which begged the question as to whether the physical relationship to each other translated to similarity in localization or function. This data was described in a recent manuscript and was followed by a press release and an LLNL best manuscript award for our team:

Sebastian A, McCool JL, Hum NR, Murugesh DK, Wilson SP, Christiansen BA and Loots GG. Single-Cell RNA-Seq Reveals Transcriptomic Heterogeneity and Post-Traumatic Osteoarthritis-Associated Early Molecular Changes in Mouse Articular Chondrocytes. ***Cells*** 2021 June 10(6):1462. DOI: 10.3390/cells10061462.

https://www.llnl.gov/news/new-research-identifies-injury-induced-molecular-changes-chondrocytes-single-cell-level

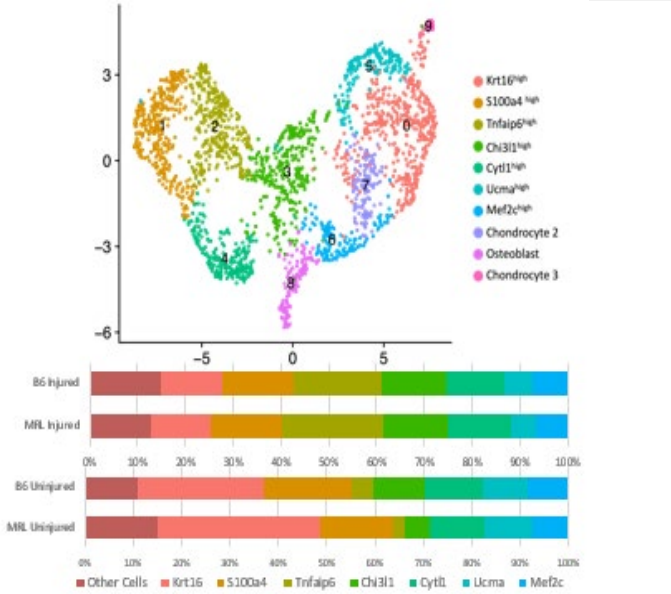


Figure 5. Merged UMAP plot representing all timepoints (injured and uninjured). Cells were clustered as 10 different chondrocyte or chondrocyte-like subpopulations for all timepoints in study: Uninjured, 2W post injury, 4W post injury (top). Histogram showing percent of total of each cluster in Uninjured and 4W post injury denoted by highly expressed genes of interest: Krt16, S100a4, Tnfiap6, Chi3l1, Cytl1, Umca and Mef2C (bottom)

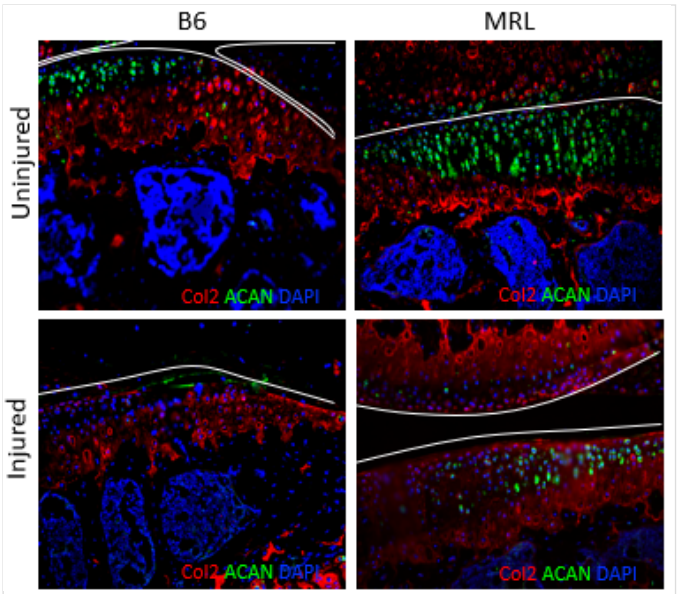


Figure 6. Immunohistochemical validation of extracellular matrix proteins in the articular cartilage. Collagen 2 (red) and Aggrecan (green) of MRL and B6 knee joints of uninjured (top row) and injured (bottom row). DAPI was used as a nuclear marker of cells (blue)

Next, we focused on comparing B6 and MRL injured and uninjured chondrocyte subpopulations (Fig. 5) and determine that MRL injured joints have a larger proportion of Krt16^{high} and Ucma^{high} chondrocytes (superficial chondrocyte markers) as well as an increased proportion of Chi3l1^{high} cells (intermediate chondrocyte marker) after injury compared to uninjured controls. The MRLs also displayed decreased expression of *S100A4* and *Tnfiap6*, two markers of fibrosis in chondrocytes, after injury. Immunohistochemical analysis of injured and uninjured joints, also confirmed that MRL mice have a thicker layer of ACAN+ cells regardless of injury (Fig. 6).

Currently, we are characterizing the injury-induced immune changes. scRNA-seq analysis of B6 mice identified 15 immune cell clusters including multiple macrophage and neutrophil clusters, T cells, NK cells, B cells and dendritic cells (Fig. 7A-B). Most striking differences were observed in macrophage clusters, with a dramatic increase in macrophage numbers in the joints by 1-day (D1) post-injury, which was significantly decrease by 15 days (D15) post-injury (Fig. 7A, C). An in-depth characterization of cells from monocyte/macrophage clusters identified 11 subclusters with distinct molecular profiles, including Ly6c2^{high} monocytes, Ccr2^{high} monocytes, Cav1^{high} differentiating monocytes/macrophages, Fcrls^{high} joint-infiltrating macrophages, antigen presenting (APC) resident macrophages with high expressing of class II MHC genes, Lyve1^{high} resident macrophages, Lcn2^{high} macrophages, interferon (*Ifn*) responsive monocytes/macrophages, proliferating cells as well as a small osteoclast cluster (Fig. 7A-B). While analyzing the immune profile of uninjured joints (D0), we identified a population of Lyve1^{high} tissue resident macrophages which robustly expressed genes such as lymphatic vessel endothelial hyaluronan receptor 1 (*Lyve1*), triggering-receptor-expressed on myeloid cells 2 (*Trem2*) and *Vsig4* (Fig.7A-B). Previous studies have identified Lyve1^{high} tissue resident macrophages in multiple tissues including heart, lungs, pancreas, bladder, blood vessels, fat tissues, and dermis and suggested that these macrophages play a key role in healing and tissue repair [30054204, 30872492, 30912746, 31481690]. Lyve1^{high} macrophages identified in the joints also expressed cartilage anabolic factors *Bmp2* and *Igf1* (Fig. 8B, red arrow) and the expression of these anabolic genes increased after injury, suggesting that Lyve1^{high} macrophages may have a cartilage protective role (Fig. 8B).

We also found that there was a dramatic increase in Cav1^{high} differentiating monocytes and Fcrls^{high} macrophages after injury, suggesting that these monocytes/macrophages could be infiltrating the joints from periphery (Fig. 8A). *Mrc1*, a marker of anti-inflammatory M2 macrophage phenotype was highly expressed in Fcrls^{high} cluster but, Cav1^{high} cluster had very few cells expressing *Mrc1*, at low level (Fig. 8B). We observed that these two clusters share a large number of genes including *Apoe, Ctsa, Ctsb* and *Ctsc* and reside close to each other on a differentiation trajectory, suggesting that Cav1^{high} monocytes could differentiate into Fcrls^{high} macrophages. Both clusters started expressing cartilage anabolic factor *Igf1* by D7 (Fig. 8B), suggesting that

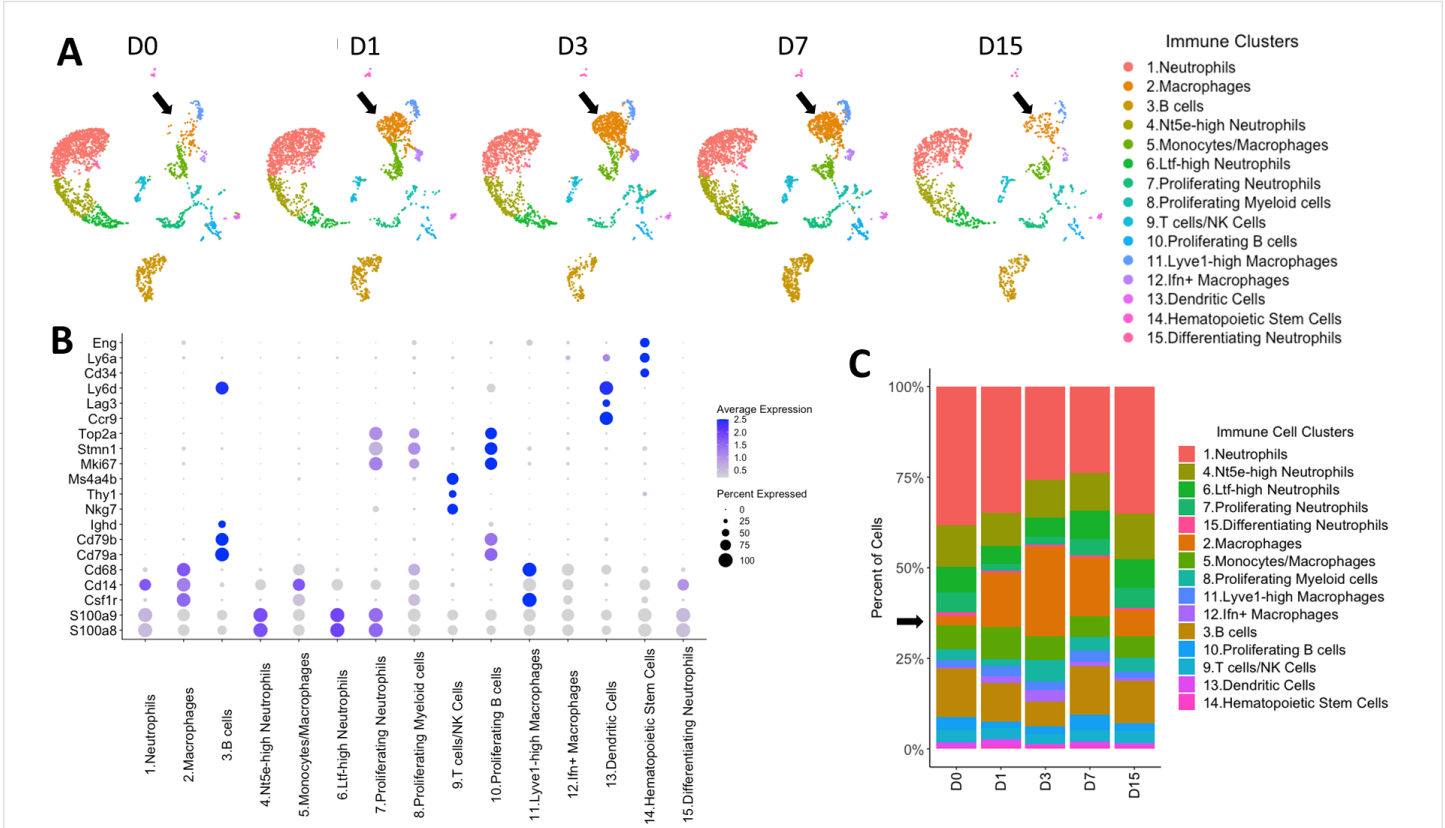


Figure 7. Single cell characterization of immune cells from young mouse knee joints. A) Immune subtypes identified in uninjured mouse knee joints (D0) and at various post-injury timepoints. Macrophage population is increased after injury (highlighted with black arrow). B) Dot plot showing the expression of selected cell type markers. Dot size represents the fraction of cells expressing a specific marker and color intensity indicates the average expression level in that cluster. C) Percentage of immune subtypes identified at various timepoints. Expanding macrophage population is highlighted with back arrow.

these monocytes/macrophage subtypes may contribute to tissue repair after injury. Interestingly, both clusters expressed *Trem2* similar to Lyve1^{high} resident macrophages and the expression increased with time (Fig. 8B). However, the transcriptome profiles of Fcrls^{high} and Cav1^{high} clusters were dramatically different from Lyve1^{high} macrophages. *Ifn* responsive monocytes also had moderate *Trem2* expression at 1- and 3-days post-injury but, did not show a significant increase in *Igf1* or other anabolic cytokine/ growth factor expression (Fig. 8B).

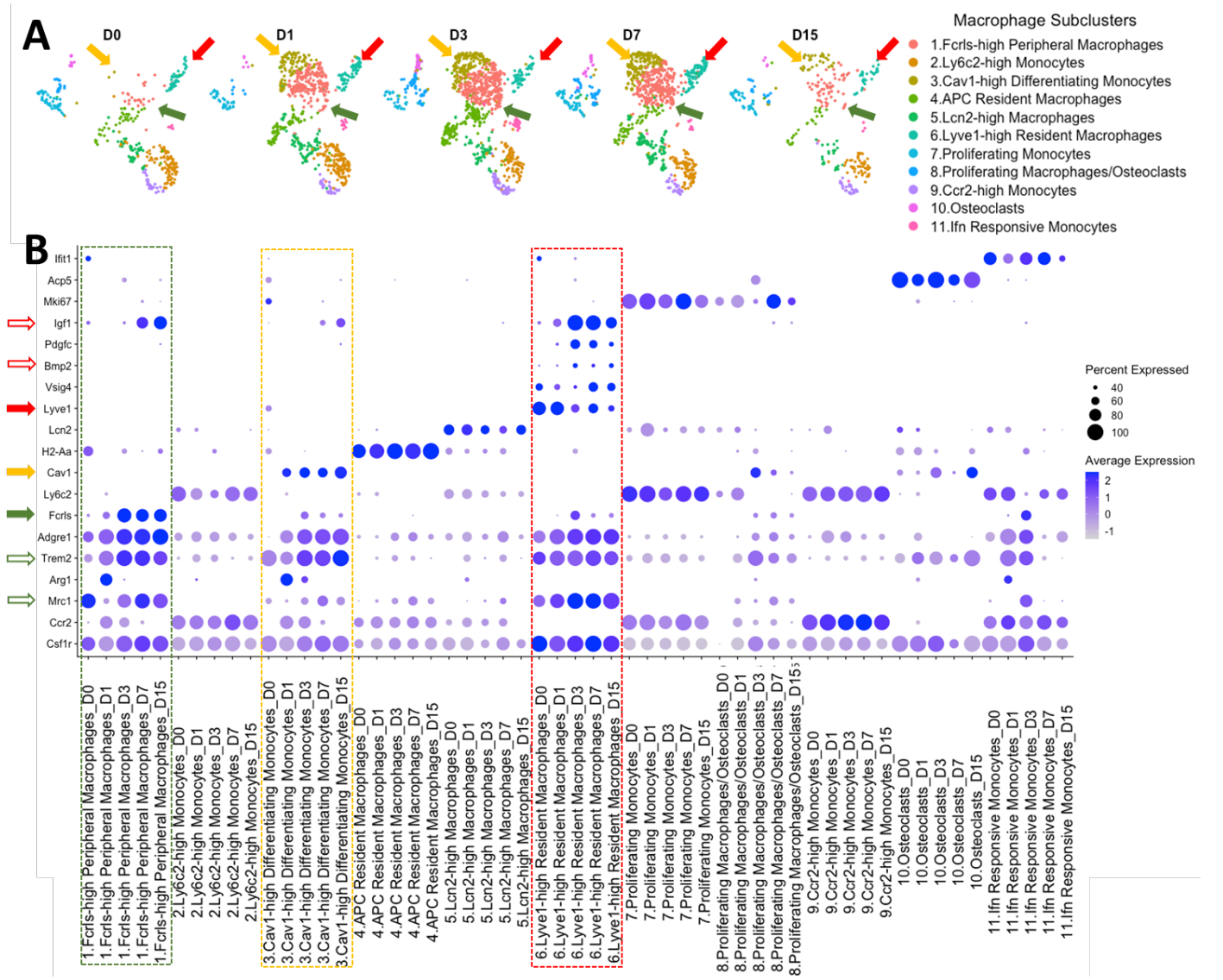


Figure 8. Injury-induced changes in macrophages from young mouse knee joints. A) Macrophage subtypes identified in uninjured 10-week-old mouse knee joints (D0) and at various post-injury timepoints. Lyve1^{high} macrophages are highlighted with red arrow, Fcrls^{high} macrophages are highlighted with green arrow and Cav1^{high} macrophages with yellow box. B) Dot plot showing the expression of selected cell type markers and genes of interest across various macrophage subtypes. Dot size represents the fraction of cells expressing a specific marker and color intensity indicates the average expression level in that cluster. Lyve1^{high} macrophages are highlighted with red box, Fcrls^{high} macrophages are highlighted with green box and Cav1^{high} macrophages with yellow box. *Lyve1*, *Vsig4* and growth factors enriched in Lyve1^{high} macrophages are highlighted with red arrow. *Fcrls*, *Trem2*, and *Mrc1* are highlighted with green arrow and *Cav1* with black arrow.

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

At UC Davis, one postdoctoral scholar (Hailey Cunningham) and one Ph.D. student (Sophie Orr) are being trained on research related to this project. Dr. Cunningham recently completed her degree in Dr. Christiansen's laboratory, and is continuing to develop her technical and professional skills while she mentors and instructs Sophie Orr (who is starting her second year as a Ph.D. student) in research methodology related to this project, including non-invasive knee injury, survival surgery, and micro-computed tomography. Sophie is currently completing the remaining graduate-level Biomedical Engineering courses required for her degree, and both trainees regularly attend weekly research seminars in the UC Davis Veterinary Orthopaedic Research

Laboratory and the Department of Biomedical Engineering. Both trainees will also continue to attend the two premier bone and cartilage national meetings, ORS and ASBMR, every year during their training. Dr. Cunningham's professional goal is to transition to a position in science policy or advocacy, and she is currently pursuing opportunities in that area. Sophie Orr's training goals are: 1.) Didactic training in biomedical engineering, orthopaedic research, and molecular and cell biology; 2.) Hands-on instruction in analysis methodology, statistical analysis, and experimental design; 3.) Professional development that leads to a career in an area related to space biology.

How were the results disseminated to communities of interest?

McCool, JL, Sebastian, A, Hum, NR, Murugesh, DK, Christiansen, BA, Loots, GG (2021). ***Single-Cell RNA Sequencing Highlights Unique Sub-populations Of Cells In The Knee Joints Of MRL/MpJ Superhealers***. Podium Presentation. Orthopaedic Research Society Annual Conference, February 12-16th 2021 – Virtual Meeting (Virtual Podium Presentation; presenter Jillian McCool)

Sebastian, A, McCool, JL, Hum, NR, Murugesh, DK, Wilson, SP, Christiansen, BA, Loots, GG (2021). ***Defining Articular Chondrocyte Heterogeneity And Injury- induced Molecular Responses At Single Cell Level.*** Podium Presentation. Orthopaedic Research Society Annual Conference, February 12-16th 2021 – Virtual Meeting (Virtual Podium Presentation; presenter Aimy Sebastian)

Abstracts Submitted during this funding period:
McCool, JL, Sebastian, A, Hum, NR, Murugesh, DK, Christiansen, BA, Loots, GG. (2021). ***Identification of Chondrocyte Sub-populations Using Single-Cell RNA Sequencing to Determine Injury Induced Cellular and Molecular Changes of C57Bl6 and MRL/MpJ Arthritic Knee Joints***. ASBMR 2021 Annual Meeting October 1-4th 2021 - San Diego, California. (In Person Plenary Poster Presentation & Oral Poster Session; presenter Jillian McCool)

- ASBMR 2021 Annual Meeting Young Investigator Travel Grant
- University of California Merced GSA Travel Award

Rios Arce ND, Murugesh DK, Hum NR, Sebastian A, Jbeily EH, Christiansen BA and Loots GG (2021). ***Type 1 Diabetes Blunts the Effects of Joint Trauma on Osteoarthritis.*** ASBMR 2021 Annual Meeting October 1-4th 2021 - San Diego, California. (In Person Plenary Poster Presentation & Oral Poster Session; presenter Naiomy Rios Arce)

- ASBMR 2021 Annual Meeting Late Breaking Travel Grant ($500 to Dr. Rios Arce)

Invited Seminar speaker, UCLA Musculoskeletal Seminar Series, Gabriela G. Loots: ***Mining OMIC Landscapes to Promote Musculoskeletal Resilience for Life. 2/10/2021*** Virtual presentation.

What do you plan to do during the next reporting period to accomplish the goals?

Our main focus for the next period will be to complete **Subtask 2**.

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Our recent manuscript,

Sebastian A, McCool JL, Hum NR, Murugesh DK, Wilson SP, Christiansen BA and Loots GG. Single-Cell RNA-Seq Reveals Transcriptomic Heterogeneity and Post-Traumatic Osteoarthritis-Associated Early Molecular Changes in Mouse Articular Chondrocytes. ***Cells*** 2021 June 10(6):1462. DOI: 10.3390/cells10061462.

Even though it has only been out for a couple of months, it already has 2 citations.

What was the impact on other disciplines?

We anticipate that the single cell RNA-seq chondrocyte methods book chapter we will publish in *Methods in Molecular Biology* book entitled "Cartilage Tissue Engineering' will become a standard protocol for purifying chondrocytes from articular cartilage in many laboratories that plan to employ single cell RNA-seq for molecular profiling.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them

Challenges due to Covid-19 Pandemic*:* Due to social distancing rules and the limited reopening of our laboratory starting in August 2020, we have had some delays in conducting larger experiments. The large animal experiments are quite laborious and require 3-5 people working side by side to harvest tissue, digest tissue, run the FACS instrument, isolate proteins and conduct quality control. Because of restrictions at LLNL, experiments had to be broken down into smaller cohorts of animals, where 2 people could manage 5-10 animals per day. Because of this slowdown in our injury and sample collection process, we have not been able to proceed with LC-MS/MS experiments that include all the groups and necessary controls to carry out a rigorous experiment. We are currently working at near full capacity as normal and social distancing restrictions were removed by UC Davis, but for most of the fiscal year;] our institutions continued to be at minimum critical operations and requested non-essential workers to shift their efforts to remote working.

Changes that had a significant impact on expenditures

The shelter in place, remote working restrictions and social distancing restrictions did not permit staff at either LLNL or UC Davis to work at the normal indicate effort levels, and supply and facility use costs were relatively low over the past year. This has resulted in a surplus of the funds. However, now that all of our staff is back in the lab, we hope to ramp up effort in the next funding period. We anticipate to fully spend the allocated funds, and to be able to complete the tasks that were delayed, but our project would greatly benefit from a no-cost extension of at least one year.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

None

Significant changes in use or care of human subjects

N/A

Significant changes in use or care of vertebrate animals.

None

Significant changes in use of biohazards and/or select agents

None

PRODUCTS:

Publications, conference papers, and presentations

Journal publications

Published Manuscripts acknowledging federal support by Grants PR180268/PR180268P1

Sebastian A, McCool JL, Hum NR, Murugesh DK, Wilson SP, Christiansen BA and Loots GG. Single-Cell RNA-Seq Reveals Transcriptomic Heterogeneity and Post-Traumatic Osteoarthritis-Associated Early

Molecular Changes in Mouse Articular Chondrocytes. ***Cells*** 2021 June 10(6):1462. DOI: 10.3390/cells10061462.

Books or other non-periodical, one-time publications.

McCool JL, Hum NR, Sebastian A, Loots GG. Isolation of Murine Articular Chondrocytes for Single Cell RNA or Bulk RNA Sequencing Analysis. *Methods in Molecular Biology* book entitled "Cartilage Tissue Engineering" Editors Prof. Martin Stoddart, Dr. Angela Armiento, Dr. Elena Della Bella. Springer. To be published in 2022.

Other publications, conference papers, and presentations

McCool, JL, Sebastian, A, Hum, NR, Murugesh, DK, Christiansen, BA, Loots, GG (2021). ***Single-Cell RNA Sequencing Highlights Unique Sub-populations Of Cells In The Knee Joints Of MRL/MpJ Superhealers***. Podium Presentation. Orthopaedic Research Society Annual Conference, February 12-16th 2021 – Virtual Meeting (Virtual Podium Presentation; presenter Jillian McCool)

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- ASBMR 2021 Annual Meeting Late Breaking Travel Grant ($500 to Dr. Rios Arce)

Website(s) or other Internet site(s)

Nothing to Report

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?

Name:	*Blaine A. Christiansen*
Project Role:	*Partnering PI*
Researcher Identifier (e.g. ORCID ID):	*ORCID ID: 0000-0002-0105-6458*
Nearest person month worked:	2.0
Contribution to Project:	*Dr. Christiansen performed non-invasive knee injuries in mice, and has performed micro-computed tomography imaging and analysis, and advised Dr. Loots's lab personnel on microCT methods*
Funding Support:	*In addition to this award, Dr. Christiansen receives funding support from the NIH – National Institute for Arthritis and Musculoskeletal and Skin Diseases, under award numbers R01 AR071459, R01 AR073772, and R01 AR075013*

Name:	*Hailey C. Cunningham*
Project Role:	*Postdoctoral Scholar*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*4.1*
Contribution to Project:	*Dr. Cunningham performed non-invasive knee injuries in mice, micro-computed tomography imaging and analysis, and statistical analysis. She also serves as the primary research mentor for Sophie Orr, a 2nd year Ph.D. student working in our lab.*
Funding Support:	*In addition to this award, Dr. Cunningham received financial support from the NIH – National Institute for Arthritis and Musculoskeletal and Skin Diseases, under award number R01 AR071459*

Name:	*Sophie Orr*
Project Role:	*Graduate Student Researcher*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*2.2*
Contribution to Project:	*Under the guidance of Dr. Cunningham, Sophie performed non-invasive knee injuries in mice and micro-computed tomography imaging and analysis of mouse knee samples.*
Funding Support:	*This grant*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

New Active Support:

Acceleration of Alzheimer's Disease Pathology Due to Osteoarthritis-Associated Inflammation
NIH / NIAMS R01 AR075013-02S1 01/01/2021 – 12/31/2021
10% effort Total Direct Costs
National Institutes of Health, NIAMS
31 Center Drive, MSC 2350, Bethesda MD 20892
The research proposed in this supplement will determine if systemic inflammation during OA progression accelerates Alzheimer's Disease (AD) progression in a genetic mouse model of AD (APP/PS1 mice), and whether this effect can be mitigated by joint unloading during the early phase post-injury. We hypothesize that OA and AD share a similar pathogenetic mechanism, and that inflammation associated with OA progression can accelerate AD progression and cognitive decline. Our specific aim is to determine whether OA-associated systemic inflammation accelerates the onset of AD progression and deterioration of cognitive function in APP/PS1 mice, and whether this acceleration can be mitigated by early phase unloading.
Role: PI
Grant Status: Active
Overlap: None

What other organizations were involved as partners?

Nothing to Report

SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Dr. Gaby G. Loots, the project PI will be submitting her report in parallel.

QUAD CHARTS:

N/A

APPENDICES:

N/A