AWARD NUMBER: W81XWH-17-1-0131

TITLE: Chemokine-Based Therapy for Post-Traumatic Osteoarthritis

PRINCIPAL INVESTIGATOR: Bouchra Edderkaoui., Ph.D.

CONTRACTING ORGANIZATI Loma Linda Veterans Association for Research and Education
Redlands, CA 92373-5181

REPORT DATE: March 2020

TYPE OF REPORT: Final

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

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. REPORT DATE | 2. REPORT TYPE | 3. DATES COVERED |
|---|---|---|
| March 2020 | Final | 05/15/2017 - 11/14/2019 |

**4. TITLE AND SUBTITLE**

**Chemokine-Based Therapy for Post-Traumatic Osteoarthritis**

**5a. CONTRACT NUMBER**
W81XWH-17-1-0131

**5b. GRANT NUMBER**
W81XWH-17-1-0131

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**
**Bouchra Edderkaoui., Ph.D.**

E-Mail: boudchra.edderkaoui@va.gov

**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

**Loma Linda Veterans Association for Research and Education**
**Redlands, CA 92373-5181**

**8. PERFORMING ORGANIZATION REPORT NUMBER**

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

U.S. Army Medical Research and Materiel 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**

Anterior cruciate ligament (ACL) is critical in providing stability of the knee joints. Injuries causing ruptures to the ACL are a common cause of knee instability and subsequent posttraumatic osteoarthritis.

Since Duffy antigen receptor for chemokines (DARC) binds inflammatory chemokines previously shown to be up-regulated in osteoarthritic knees, we proposed to analyze the role of DARC on the development of PTOA in the present study.

In addition to MCP-1 and RANTES, DARC binds to other chemokines previously shown to be present in OA knees such as CXCL1 and CXCL5, and which are known to be involved in neutrophil migration. Thus, to determine the role of these chemokines in PTOA development, we evaluated the effect of ACL injury on their mRNA levels. We predicted that expression of these chemokines would correlate with the level of inflammation and the magnitude of catabolic effects on cartilage and subchondral bone that occur in response to injury. At three days post ACL injury, both chemokines were found to be increased in response to knee injury, confirming the recruitment of neutrophils to the knee joints in response to ACL injury. No difference was observed in the expression of Cxcl1 or Cxcl5 between the two lines of mice suggesting that post-ACL injury inflammation was not affected by Darc deficiency.

To determine whether interfering with chemokine-DARC interaction will inhibit post-ACL injury inflammation, injured knees were treated with neutralizing antibodies against DARC or IgG control, at the knee joints. Then, the expression level of one of DARC ligands which is also one of the major inflammatory chemokines and the expression of *Mmp3* were measured. The mRNA levels of *Mcp-1* and Mmp3 were significantly greater in the injured knees compared to control un-injured knees. However, no difference in the expression of *Mcp-1* or *Mmp3* was found between DARC-Ab and IgG treated knees at three day-post ACL injury.

**15. SUBJECT TERMS**

ACL, DARC, Inflammation, PTOA

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

## Table of Contents

**Page #**

## A. INTRODUCTION

The anterior cruciate ligament (ACL) connects the femur to the tibia at the knee joint and is critical in providing stability of the knee. Injuries causing ruptures to the ACL are a common cause of knee instability and subsequent posttraumatic osteoarthritis (PTOA). US military personnel suffer from ACL injury at a rate 10 times that of the civilian population. In addition, even though ACL injuries can be surgically repaired, following reconstructive ACL surgery, 35% of patients will develop tibio-femoral osteoarthritis (OA) (Svoboda, 2014). Clearly, ligament damage, especially to the ACL is a significant factor in the initiation and progression of PTOA, and merits further studies in order to develop effective therapeutic interventions.

**Hypothese**s. Three hypotheses were proposed in this study: (**1**) Anterior cruciate ligament (ACL) injury/rupture induces cytokine secretion in the synovial space, leading to increased chemokine expression; (**2**) then, through DARC-dependent chemokine transcytosis will increase chemokine secretion, in turn promoting the recruitment of inflammatory cells to the joint space, which will lead to cartilage degradation and PTOA; and (**3**) blocking the function of DARC will inhibit chemokine binding to DARC after ACL injury, reducing the recruitment of inflammatory cells to the joint space, and reducing or preventing the onset and/or progression of PTOA.

**Objectives.** Developing treatments for PTOA requires an understanding of the underlying molecular mechanisms. For this purpose, we proposed to first identify those chemokines that bind to DARC, and whose expression is induced in response to ACL injury. Time course experiments will identify the temporal relationship between DARC-chemokine interactions and the development of PTOA pathology. Together, these studies will provide critical information about the molecular mechanisms that contribute to inflammation and cartilage damage, and will identify when chemokine-DARC interaction occurs. This will aid in the design of the second series of experiments to evaluate, in a mouse model of PTOA that mimics joint injury in humans, the effect of local administration of DARC neutralizing antibodies on PTOA development, as well as potentially identifying other targets that may be amenable for therapeutic intervention.

Therefore, we proposed two specific aims to confirm the above hypotheses:

**Specific Aim 1.** Identify the chemokines induced and the cells that migrated to the synovial fluid in response to knee injury during the development of PTOA

**Specific Aim 2.** Develop a strategy to locally reduce or inhibit post-ACL injury mediated inflammation and development of PTOA by direct administration of anti-DARC antibodies to the injured knee

## B. BODY

### Progress report during the first year of the funding period

1. **Specific Aim 1.** Identify the chemokines induced and the cells that migrated to the synovial fluid in response to knee injury during the development of PTOA
   **a**. **Gene expression profile in response to ACL injury caused by axial loading**
   - **Animal Model.** Optimize the conditions for axial loading to induce ACL tear/rupture. 10-week old C57/B6 wild type (WT) and Darc-KO mice were used during this funding period.

Mice were anesthetized by isoflurane inhalation. Using an Instron Hydraulic machine, the right leg was subjected to a preload of 1-2N applied to the knee, followed by a single dynamic axial compressive load of 15 N (at a 40 N/s loading rate) as previously described (Christiansen et al., 2012). This was successful to induce knee injury, characterized by a discontinuity in the force-displacement curve at 12N (**Fig. 1**). Subcutaneous injection of buprenorphine analgesia (0.5 μg/g body weight) was administered to each animal after injury. The animals were allowed unrestricted movement following injury. The left knee served as contralateral control.

Animals were sacrificed at different time points post-ACL injury. Tissues were collected by excising a region extending 1-2 mm above and below the middle of the knee joint, with samples snap frozen in liquid nitrogen, and stored at -80°C. For RNA extraction, samples were pulverized in liquid nitrogen; total RNA was isolated using Trizol and RNeasy kit (Qiagen) and processed for real-time-PCR. Real-time quantitative PCR was performed using the Applied Biosystems ViiA7 RT-PCR systems instrument, and the SYBR Green PCR kit from Applied Biosystems Inc.

- **Gene expression profile post-axial loading**
  - <u>Gene expression profile at 1, 3, 7 and 21 days post-ACL injury</u>

The expression levels of the major inflammatory cytokines as well as the chemokines that bind to DARC and the matrix degrading enzymes were evaluated at different time points post-axial loading (AL), using Real Time PCR as previously described (Edderkaoui et al., 2007).

In samples collected from knees from WT and *Darc*-KO mice, we observed a 7- 8-fold increase in the mRNA level of *Il-6* at one-day post-knee injury (**Fig. 2**). In addition, the mRNA levels of *Il-1β* and *Cd14* were similarly increased in both lines of mice (**Fig. 2**).

DARC binds to several chemokines previously shown to be present in OA knees (e.g., IL-8, CXCL1, MCP-1/CCL2 and RANTES/CCL5) (Bay-Jensen et al., 2015), and which are known to be involved on inflammatory cell migration. Thus, to determine the role of these chemokines in PTOA development, we first evaluated the change in their mRNA levels in response to AL. We predicted that expression of these chemokines would correlate with the level of inflammation in the knee, and the magnitude of catabolic effects on cartilage and subchondral bone that occur in response to injury.

At one day-post knee injury (**Fig. 3A**), no significant increase was observed in the mRNA level of *Rantes* in any mouse line. However, the expression of *Mcp-1* was induced in experimentally injured knees collected from both WT and *Darc*-KO mice, but the magnitude of increase was significantly greater in *Darc*-KO mice compared to WT mice. This could in part be

due to the difference in basal levels of *Mcp-1* mRNA, which were higher in *Darc*-KO mice as compared to WT mice.

Since our previous data, from a different animal model of PTOA, showed the level of *Ccl21* increasing during osteoarthritis development, we evaluated the mRNA level of *Ccl21* at two different time points. While a small reduction in the expression of *Ccl21* was found in the injured knees collected from WT mice as compared to non-injured knees, a significant reduction in the mRNA level of *Ccl21* was observed in the injured knees collected form *Darc*-KO mice at one-day post-knee injury (**Fig. 3A**).

At three days post-AL, we have evaluated the expression of *Ccl21* and 4 chemokines that bind to DARC; MCP-1 (CCL2), RANTES (CCL5), CXCL1 and CXCL5. Among the chemokines tested, *Ccl21* (**Fig. 3B**), *Cxcl1* and *Cxcl5* (**Fig. 4**) mRNA levels were significantly increased in the injured knees compared to un-injured knees in both lines of mice. A slight change in *Mcp-1* mRNA level was observed in the injured knees compared to un-injured knees from WT mice, but no difference in *Rantes* mRNA level was observed between the injured and un-injured knees at this time point. At seven days post-ACL injury, the mRNA level of *Mcp-1* was significantly increased in both WT and KO mice, but no change in the expression of *Rantes* was observed in response to ACL injury and no difference was observed in the expression level of *Mcp-1* between the two lines of mice at this time point (**Fig. 3C**).

The mRNA levels of three matrix metalloproteinases known for their catabolic effects on the extracellular matrix, MMP3, MMP9 and 13, were evaluated at one-, three- and seven- days post-ACL injury. Only *Mmp3* showed significant increase in mRNA levels when compared to unloaded knees in both lines of mice (**Fig. 5**) from day one to day 7 post-ACL injury. However, the magnitude of increase in the mRNA level was less in *Darc*-KO mice than was observed in WT mice at day one (**Fig. 5A**). Again, it is possible that this could be due to lower basal levels of *Mmp3* mRNA in *Darc*-KO compared to the WT mice.

It has been reported that abnormal mechanical stress due to knee destabilization caused by ACL injury induces osteophyte formation (Hsia et al., 2017). Since, the formation of osteophytes starts with an abnormal chondrogenesis, and since the PTOA model used in this study, has been shown to induce changes in chondrocyte physiology within four weeks post-injury in mice (Christiansen BA et al., 2012), we have evaluated the expression pattern of chondrocyte proliferation and differentiation markers at three week-post injury. The mRNA level of Collagen type II (*Col2*), *Col 10* and aggrecan (*Acan*) significantly increased in injured knees of both WT and KO mice compared to un-injured knees (**Fig. 6**), suggesting that ACL injury causes an early osteophyte formation that was not affected by the lack of *Darc* expression.

**b. <u>Histologic assessment of knee joint after meniscectomy</u>**

Injured and un-injured knee joints were collected, and muscle tissue was removed. The knees were fixed for one day in 10% buffered formalin, decalcified in Formical 4 for one day at room temperature and embedded in paraffin. Then, 5 µm sections were taken at 100-µm intervals

from the posterior to anterior side of the knee joints. Slides were stained with hematoxylin and Safranin-O/Fast green to assess general morphology, inflammation and matrix proteoglycans.

Immunostaining was performed to identify the cells that migrated to the injured knees, and evaluate MMPs secreted in response to ACL. Antibodies against CD4, CCR7, CD19 and MMP13 were used in this study.

At 3 days post knee injury, the knee joints showed infiltration of several inflammatory cells both at the connection ACL – femoral condyle and at to the synovium (**Fig. 7**). Immunostaining was performed to identify the cells that migrated to the injured knees in response to AL, we have used antibodies that bind to T cell subset CD4 and one of the major markers of T cells, CCR7, as well as CD19 which is expressed on virtually all B cells with the exception of plasma cells. While a huge number of CD4 and CCR7 positive cells was observed at the connection ACL – femoral condyle and at to the synovium of the injured knees, these cells were mostly only found at bone marrow in control un-injured knees (**Fig. 8**). With CD19 antibodies, we did not see any staining at the injured knees (data not shown), suggesting, no early infiltration of B cells in response to ACL injury.

Immunostaining using antibodies against MMP13 showed significant increase in the level of MMP13 in articular cartilage from the injured knees compared to uninjured knees, which was not revealed by qPCR of the whole knees. These discrepancies between data from qPCR and immunostaining could be explained because, mRNA used for gene expression evaluation was isolated from the whole knee which made it difficult to detect the changes in *Mmp13* expression in the articular cartilage, or because ACL injury altered the translation of MMP13 but did not significantly affect the transcription of *Mmp1*3 gene.

Since gene expression profiles showed increased expression of some chondrocyte differentiation markers (**Fig. 6**) at three weeks post ACL injury, we predicted an early osteophyte formation caused by ACL injury, so we analyzed the histology sections stained with Safranin-O/Fast Green that were collected from mice animals at two-weeks post ACL injury (**Fig. 8B** and **C**). Fibrous tissue (FT) was obvious around the injured knees from both lines of mice. The injured knees showed synovial thickening and a swelling like phenotype. Safranin-o staining appeared at the side of the femur condyle and tibia plate (**Fig. 8B** and **C**) as well as at the connection of the menisci and the cruciate ligaments with the tibia bone (**Fig. 8C**) in the injured knees. The collateral un-loaded knees showed a smooth articular cartilage, smooth medial collateral and smooth lateral collateral ligament, no fibrous tissue was observed in un-loaded knees, no safranin-o staining was observed at the connection of the menisci and cruciate ligament with tibia bone (**Fig. 9A**). The apparition of safranin-o staining is a sign of osteophyte formation and explains the increased levels of type 2 collagen (Col. 2), Collagen X (Col. 10) and Aggrecan (Acan) detected at three weeks post-ACL injury with qPCR (**Fig. 6**).

At 8-weeks post injury (**Fig. 9E** and **F**), specimens from injured knees showed a significant loss of articular cartilage, not only at the superficial zone but also in the calcified cartilage layer. There was no significant difference in cartilage loss between the two lines of mice at this time point (**Fig. 9**). In contrast, in the sham un-injured knees, the superficial layer of cartilage was smooth, and no disruption of surface integrity was observed. The cartilage matrix was well stained

with Safranin-O. Preservation of Safranin-O staining and chondrocytes in calcified cartilage layer and smooth bony trabeculum was observed in subchondral layer (**Fig. 9D**).

2. **Specific Aim 2.** Develop a strategy to locally reduce or inhibit post-ACL injury mediated inflammation and development of PTOA by direct administration of anti-DARC antibodies to the injured knee

In this specific aim 2, we tested the effect of local blockade of DARC function on inflammation and Mmp gene expression post ACL injury. Intra-articular injection of the neutralizing antibody against DARC or IgG control was performed at one day-post ACL injury, we tested 2 doses; 0.4 μg and 4 μg DARC-antibody (Ab) that was diluted in PBS, we are only presenting the data from 4 μg DARC-Ab, because 0.4 ug DARC-Ab did not affect post ACL inflammation. The animals were sacrificed at three days post ACL injury.

To determine whether interfering with chemokine-DARC interaction will inhibit inflammation, we have evaluated the expression level of two of DARC chemokine ligands (Cxcl5 and Mcp-1/Ccl2) and *Ccl21*. In both DARC-Ab and IgG treated groups, the mRNA level of *Mcp-1* and Ccl21 was significantly greater in the injured knees compared to control un-injured knees (**Fig. 10**). However, CXCL5 that regulates neutrophil homeostasis and is known for its implication in connective tissue remodeling, was found to be down-regulated in the injured knees treated with DARC-Ab compared to un-injured and compared to injured knees treated with the control IgG.

We have also compared the response of two major matrix metalloproteinase genes to ACL injury, in the presence and in the absence of DARC-Ab. The mRNA level of *Mmp3* was greater in the injured knees compared to un-loaded knees in both groups, and no difference in the expression of *Mmp3* was found between DARC-Ab treated knees and IgG treated knees.

## C. KEY RESEARCH ACCOMPLISHMENTS DURING THE FUNDING PERIOD OF THIS PROJECT

We have made the following achievements in this research project:

- We have optimized the conditions of the axial loading model to induce ACL injury
- We have evaluated the expression of pro-inflammatory cytokines and chemokines as well as the expression matrix degrading enzymes at different time points post-ACL injury.
- We observed a significant increase in the expression of *Il-6*, Il-1β as well as CD14 at one-day post-ACL injury in both *Darc*-KO and WT mice; small but not significant differences in the expression were observed between the two lines of mice in response to knee ACL injury.
- We have evaluated the change in the expression of the two chemokines that bind to DARC and in the expression of *Ccl21* as well as the two major effectors of articular cartilage catabolism, *Mmp3* and *Mmp13* at one-, three- and seven-days post ACL injury.

- With qPCR, the greatest change in gene expression was found in the expression of *Mmp3* as early as one-day post ACL injury.
- *Ccl21* mRNA level was induced later on in the injured knees and became significantly greater compared to un-injured knees, at three days post ACL injury in both lines of mice.
- We have analyzed the expression levels of chondrocyte proliferation and differentiation markers at the knee joints, three weeks post ACL-injury in both lines of mice.
- mRNA level of Collagen type II (*Col2*), *Col 10* and aggrecan (*Acan*) significantly increased in injured knees of both WT and KO mice compared to un-injured knees suggesting that ACL injury causes an early osteophyte formation that was not affected by the lack of *Darc* expression.
- Our examination of the sections prepared from knees collected at 3 days post-knee injury revealed inflammatory secretions and the presence of abundant CD4 and CCR7 positive cells in the synovium around the injured knees and at the connection of cruciate ligaments.
- In addition, immunostaining of histology sections collected from injured knees, showed significant increase in MMP13 at the articular cartilage of injured knees compared to control knees, at three days post injury.
- Our examination of the histology sections prepared from the knees collected at two weeks post-ACL injury, revealed synovial thickening and knee swelling as well as a starting of osteophyte formation.
- Our evaluation of the sections prepared from knees collected at 8 weeks post-knee injury revealed the presence of chondrocyte loss, and a loss of Safranin staining, which extended into the radial zone. While the sections derived from the control unloaded knees showed smooth superficial layer of cartilage, no disruption of surface integrity was observed.
- To block the function of DARC locally at the knee joints, we have tested 2 doses; 0.4 μg and 4 μg DARC-antibody (Ab). At one day-post ACL injury, DARC-Ab or IgG control were injected at the injured knees. Then, animals were sacrificed at three days post ACL injury.
- we have evaluated the expression level of *Mcp-1*, *Cxcl5*, *Mmp3* and *Mmp13* in both control and DARC-Ab treated mice at three days post ACL injury.
- In both group of mice, the mRNA level of *Mcp-1* was significantly greater in the injured knees compared to control un-injured knees, no difference was observed between the group treated with DARC-Ab and control treated group.
- The mRNA level of *Mmp3* was greater in the injured knees compared to un-loaded knee, and no significant difference in the expression of *Mmp3* was found between DARC-Ab treated knees and IgG treated knees.
- Only *Cxcl5* showed significant reduction in the expression in the injured knees treated with DARC-Ab compared to control injured knees.

## D. CONCLUSION

Knee ACL injury caused by axial loading induces the expression of multiple genes at different time points. The expression of Il-6 and Il-1β, the major pro-inflammatory cytokines, and Mmp3, known for its role in cartilage degradation, was induced as early as one day post-knee injury, but the expression of the Ccl21 chemokine was induced later at the injured knees, with significantly increased mRNA level evident at three days post injury. Furthermore, *Mmp3* mRNA levels remained significantly elevated in the injured knees compared to control knees during the first week post-knee injury in both lines of mice. Interestingly, no difference was observed in the response of pro-inflammatory chemokines between WT mice and *Darc*-KO mice suggesting that DARC is not involved in the inflammation induced by knee injury. Furthermore, no significant difference was observed in cartilage loss between the two lines of mice at 2 or 8-weeks post-knee injury.

The local blockade of DARC function, by injection of DARC neutralizing antibody at the injured knee, one day post injury caused significant reduction in the expression of Cxcl5. Since no effect was observed on any of the matrix degrading enzymes tested in this study, we concluded that DARC does not play an important role on PTOA development post ACL injury.

**E. INVENTIONS, PATENTS AND LICENSES:** Nothing to report

**F. REPORTABLE OUTCOMES:**

- ACL injury induced the expression of Il-6, Il-1β, CD14 and Mmp3 as early as one day post injury.
- CCL21 expression was induced later with mRNA levels significantly higher in injured knees compared un-injured knees at three days post knee injury.
- ACL injury induced the recruitment of CD4 T cell subsets that express CCR7.
- ACL injury induced osteophyte formation as early as two weeks post ACL injury.
- Osteophyte formation starts with chondrogenesis around the injured knee joints.
- At 8-weeks post knee injury, a great loss of articular cartilage, which extended into the radial zone was observed in both lines of mice.
- Total body *Darc* deficiency did not affect post -ACL inflammation injury nor PTOA development.
- ACL injury induced the expression of two other chemokines (CXCL1 and CXCL5), that binds DARC and are responsible for neutrophil migration, in the injured knees post ACL injury.
- Local blockade of DARC function at the knee joints altered the expression of Cxcl5 but did not affect the expression of *Mmp3,* suggesting that DARC does not play an important role on PTOA development post ACL injury.

**G. OTHER ACHIEVEMENTS:** Nothing to report

**H. REFERENCES:**

**.** Svoboda SJ. 2014 ACL Injury and Posttraumatic Osteoarthritis. Clin Sports Med.;33(4):633-640.

**.** Christiansen BA, Anderson MJ, Lee CA, Williams JC, Yik JH, Haudenschild DR. 2012 Musculoskeletal changes following non-invasive knee injury using a novel mouse model of posttraumatic osteoarthritis. Osteoarthritis Cartilage.;20(7):773-82.

**.** Bay-Jensen AC, Reker D, Kjelgaard-Petersen CF, Mobasheri A, Karsdal MA, Ladel C, Henrotin Y, Thudium CS. 2016 Osteoarthritis year in review 2015: soluble biomarkers and the BIPED criteria. Osteoarthritis Cartilage.;24(1):9-20.

**.** Edderkaoui B, Baylink DJ, Beamer WG, Wergedal JE, Porte R, Chaudhuri A, Mohan S. 2007 Identification of mouse Duffy antigen receptor for chemokines (Darc) as a BMD QTL gene. Genome Res. 2007 May;17(5):577-85.

**.** Hsia AW, Anderson MJ, Heffner MA, Lagmay EP, Zavodovskaya R, Christiansen BA. Osteophyte formation after ACL rupture in mice is associated with joint restabilization and loss of range of motion. J Orthop Res. 2017 Mar;35(3):466-473.

## I. FIGURE LEGENDS

**Figure 1. ACL injury during tibial compression loading** identified by a release of compressive force during the loading cycle, with a continued increase in actuator displacement. X axis represents the time in milliseconds, the Y axis represents the loading force in Newton.

**Figure 2. mRNA expression levels of the two major inflammatory genes; *Il6* and *Il-1β* and monocyte marker *Cd14* at the knee joints at one day post-ACL injury.** We collected knees from both control unloaded knees (left knees) and loaded knees (right knees). Data are presented as mean of fold changes compared to WT unloaded knees ± St. dev., n=4 and **P*<0.05 vs WT left knees. Rk., for right knees, and Lk., for left knees.

**Figure 3. mRNA expression levels of the two inflammatory chemokines that bind to DARC and *Ccl21* at different time points post-ACL injury. One day (A), three days (B) and seven days (C ) post-ACL injury.** We collected knees from both sham unloaded knees (left knees) and loaded knees (right knees). Data are presented as mean of fold changes compared to WT unloaded knees ± St. dev., n=4 and **P*<0.05 vs WT left unloaded knees. #P<0.05 comparing the expression at the right knees between the two lines of mice. $P<0.05 comparing the basal expression between the two lines of mice at the left knee. Rk., for right knees, and Lk., for left knees.

**Figure 4. mRNA expression levels of chemokines; Cxcl1 and Cxcl5 at the knee joints of *Darc*-KO and WT mice, at three days post-ACL injury.** We collected knees from both control unloaded knees (left knees) and loaded knees (right knees). Data are presented as mean fold changes vs WT unloaded knees ± St. dev., n=4 and *P<0.05 vs WT left knees. Rk., for right knees, and Lk., for left knees.

**Figure 5. mRNA expression levels of the three major matrix metalloproteinases, *Mmp-3*, *Mmp-9* and *Mmp-13* at the knee joints, at one (A), three (B) and seven (C ) days post-ACL injury.** We collected knees from both sham unloaded knees (left knees) and loaded knees (right knees), data are presented as mean fold changes compared to WT unloaded knees ± St. dev., n=4 and **P*<0.05 vs WT left unloaded knees. #P<0.05 comparing the expression at the right knees between the two lines of mice. Rk., for right knee, and Lk., for left knee.

**Figure 6. mRNA expression levels of three markers of chondrogenesis in the knee joints of both WT and Darc-KO mice, at three weeks post-ACL injury.** We collected knees from both sham unloaded knees (left knees) and loaded knees (right knees). Collagen type 2 alpha 1 (Col 2), Collagen, type x, alpha-1 (Col 10) and aggrecan (Acan). Data are presented as mean of fold changes in right knees compared to WT unloaded knees (left knee) ± St. dev., n=5 and *P<0.05 vs WT left unloaded knees. #P<0.05 comparing the expression at the right knees between the two lines of mice. Rk., for right knees, and Lk., for left knees.

**Figure 7. Representative images from histology sections of intact knees (A) and injured (B) knees from WT mice, at three days-post ACL injury. A. 1.** Shows intact medial meniscus. **B.1.**

Shows injured medial meniscus with inflammatory cells (red arrow) and synovitis **A. 2.** Shows intact ligaments with no inflammation. **B.2.** Shows ruptured ACL with abundant inflammatory cells (red arrow). **A.3**. and **B.3.** represent the collateral sides of the un-injured knee and the injured knee, respectively. 1, 2 and 3 squares represent the areas that were visualized using microscope 4x lens.

**Figure 8. Representative images from histology sections of intact knee (A and C) and injured (B and D) knees showing an increase in CCR7 and CD4 positive cells, at three days-post ACL injury. A.,** and **C.** Show intact cruciate ligaments with no inflammation, no synovitis. **B**., and **D**. Show injured ACL with inflammatory cells and synovitis (brown color). Immunostaining was performed using antibodies against mouse CD4 and CCR7. F. femur, T. Tiba, and CLC. Cruciate ligament connection.

**Figure 9. Representative images from histology sections of intact knees (A and D) and injured knees (B, C, E and F), at two (A, B, C) and eight weeks-post ACL injury from both WT and *Darc*-KO mice. A. and D.,** The medial sides of the unloaded left knees, collected from WT mice, show smooth ligaments connecting femur bone to tibia bone (thick arrow) and smooth cruciate ligament (CL, thick arrow), no fibrous tissue was observed. **B.** The medial side of the right knee from WT mice, after ACL injury. The image shows fibrous tissue (FT), swelling like phenotype, and cartilage synthesis as evidenced by safranin-o staining (thin black arrows). **C.** The medial side of the right knee from Darc-KO mice, after ACL injury. The image shows fibrous tissue (FT), swelling like phenotype, and cartilage synthesis as evidenced by safranin-o staining (thin black arrows). **E** and **F**. Injured knees from WT and *Darc*-KO mice showing irregularities, and severe loss of Safranin-O staining at the articular cartilage (blue arrows), mostly at the medial. F. for femur, T. for tibia. CL. for cruciate ligament. FT. for fibrous tissue. Sections were stained with Safranin-O/Fast Green/hematoxylin and observed using microscope lens 1.25 x.

**Figure 10. mRNA expression level of three major inflammatory chemokines (A) and two matrix degrading enzymes (B) at the knee joints of WT mice treated with either DARC-antibody or IgG control, at three days post-ACL injury.** We collected knees from both control unloaded knees (left knees) and loaded knees (right knees) from both DARC-Ab and IgG treated mice. Data are presented as mean fold changes compared to the unloaded left knees collected from WT mice injected with IgG ± St. dev., n=4-6 and *$P<0.05$ vs left knees from the animals injected with IgG control, #$P<0.05$ injured knees treated with DARC-Ab compared to injured knees treated with IgG control. Rk., for right knees, and Lk., for left knees

**Fig. 1**

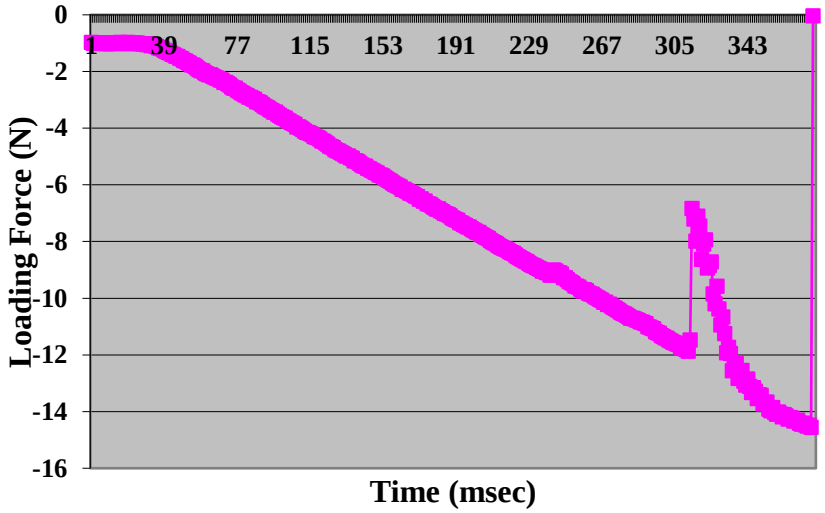

0
-2
-4
-6
-8
-10
-12
-14
-16
1
39
77
115
153
191
229
267
305
343
Loading Force (N)
Time (msec)

**Fig. 2**

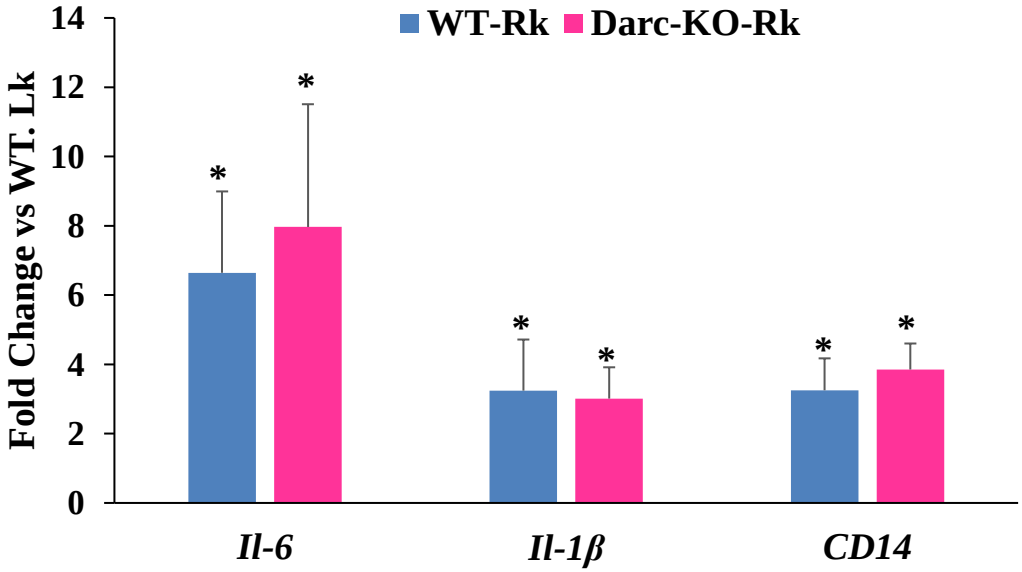

WT-Rk
Darc-KO-Rk
Fold Change vs WT. Lk
14
12
10
8
6
4
2
0
*
Il-6
Il-1β
CD14

**Fig. 3**

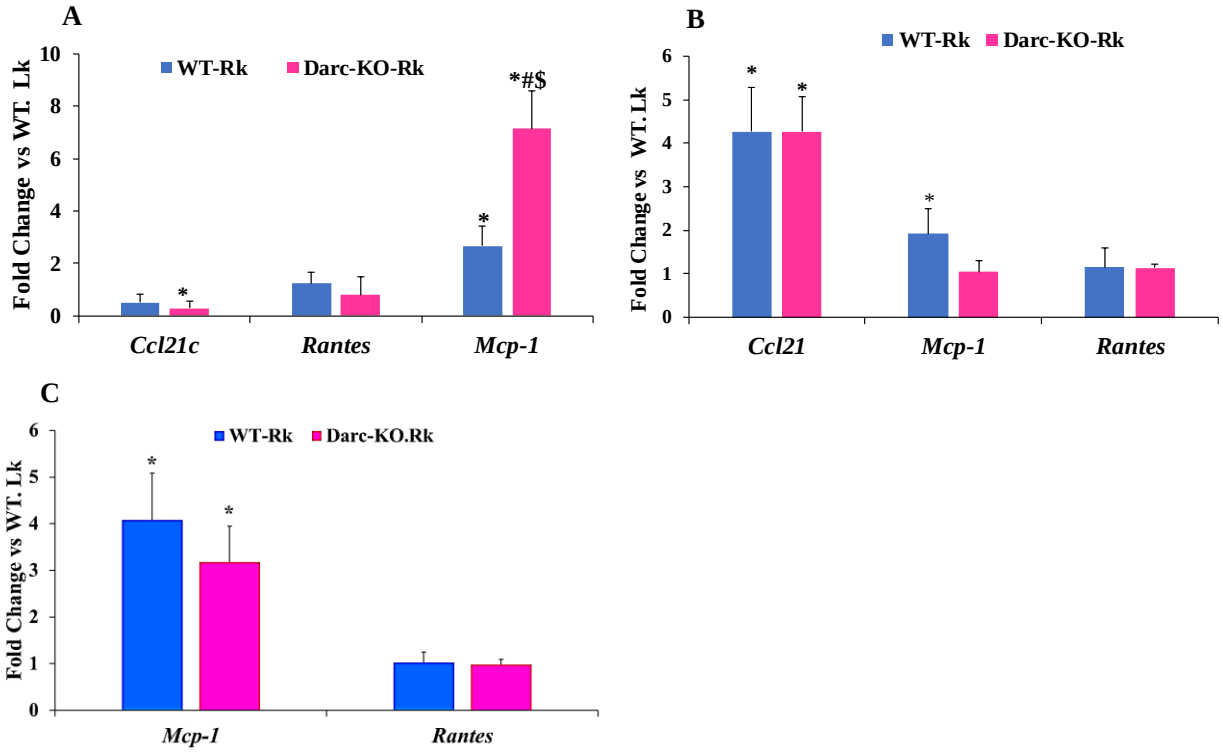

A
WT-Rk
Darc-KO-Rk
Fold Change vs WT. Lk
10
8
6
4
2
0
*
*
*
*#$
Ccl21c
Rantes
Mcp-1
B
WT-Rk
Darc-KO-Rk
Fold Change vs WT. Lk
6
5
4
3
2
1
0
*
*
*
Ccl21
Mcp-1
Rantes
C
WT-Rk
Darc-KO.Rk
Fold Change vs WT. Lk
6
5
4
3
2
1
0
*
*
Mcp-1
Rantes

**Fig. 4**

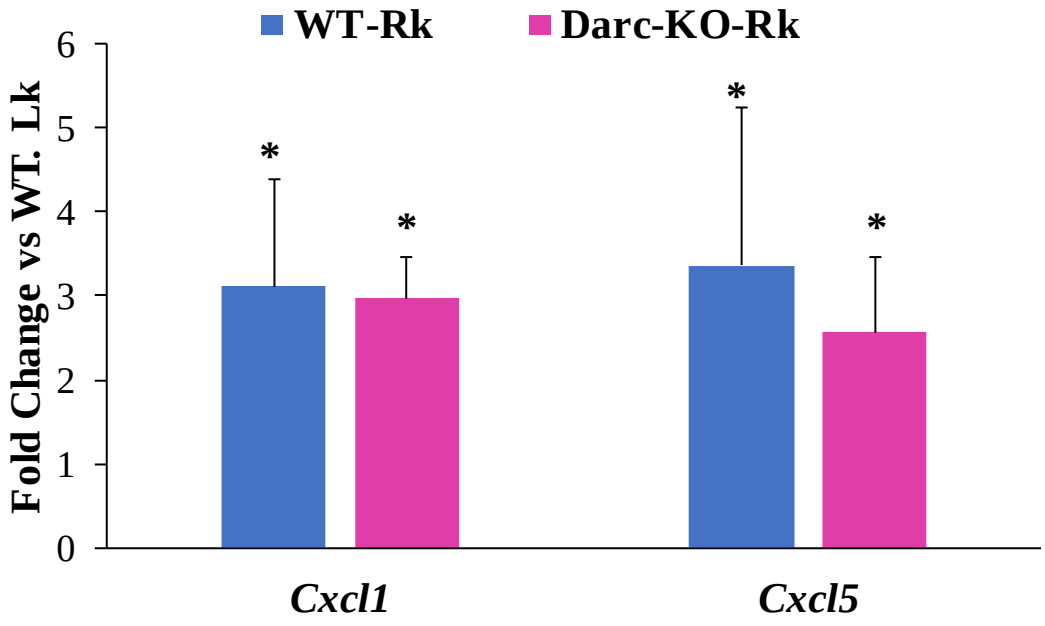

WT-Rk
Darc-KO-Rk
Fold Change vs WT. Lk
6
5
4
3
2
1
0
*
*
*
*
Cxcl1
Cxcl5

**Fig. 5**

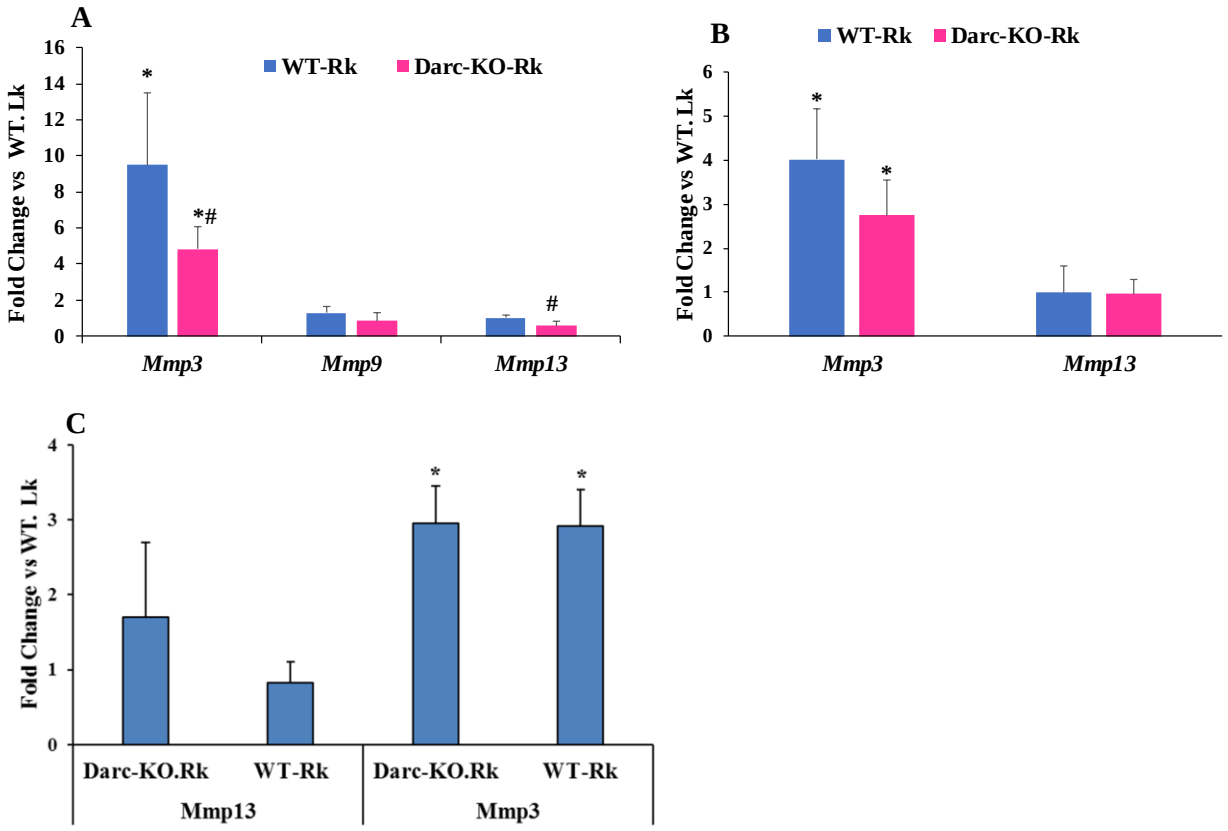

A
WT-Rk
Darc-KO-Rk
Fold Change vs WT. Lk
16
14
12
10
8
6
4
2
0
*
*#
#
Mmp3
Mmp9
Mmp13
B
WT-Rk
Darc-KO-Rk
Fold Change vs WT. Lk
6
5
4
3
2
1
0
*
*
Mmp3
Mmp13
C
Fold Change vs WT. Lk
4
3
2
1
0
*
*
Darc-KO.Rk
WT-Rk
Darc-KO.Rk
WT-Rk
Mmp13
Mmp3

**Fig. 6**

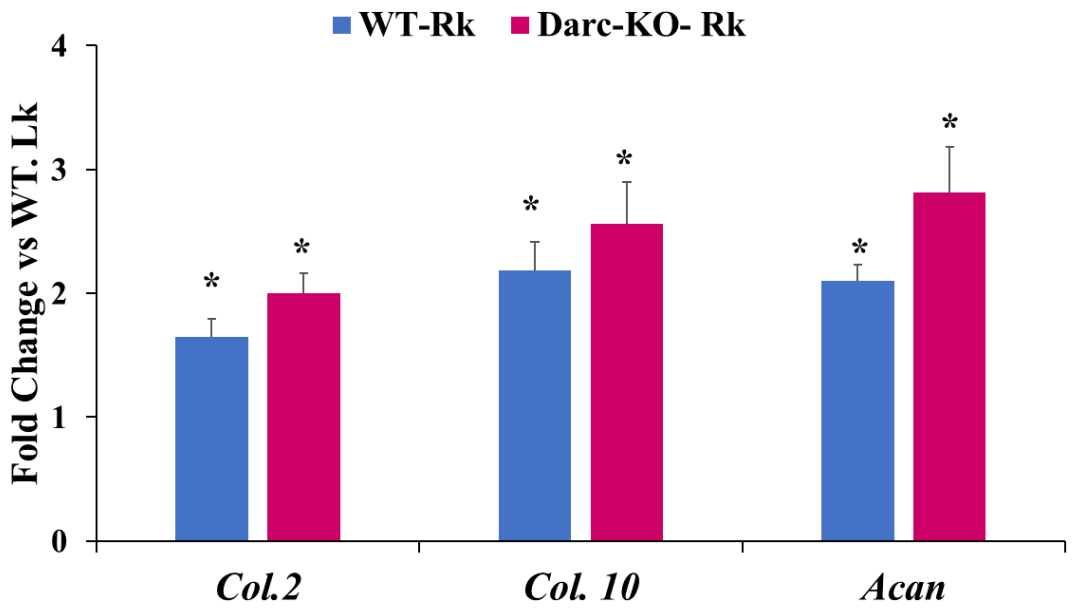

WT-Rk
Darc-KO- Rk
Fold Change vs WT. Lk
0
1
2
3
4
*
Col.2
Col. 10
Acan

**Fig. 7**

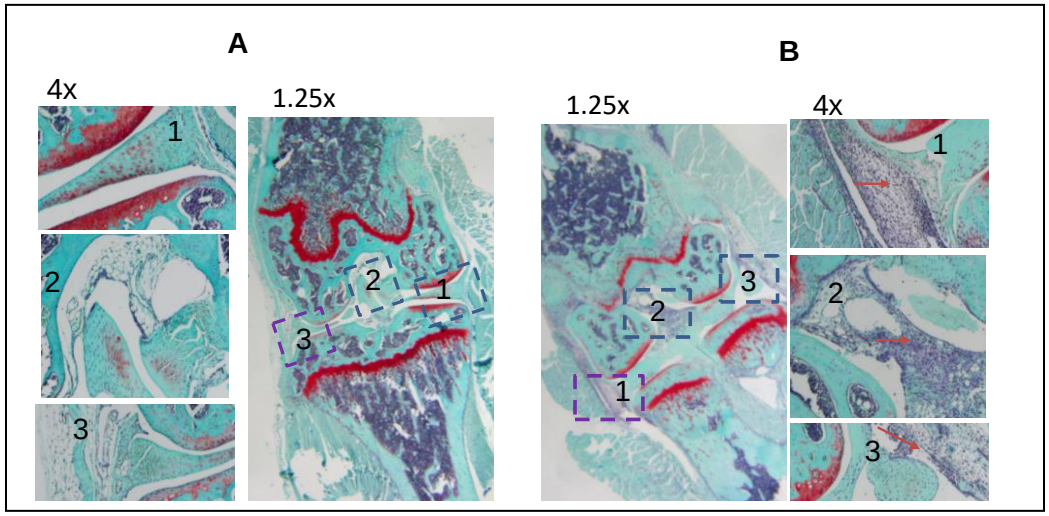

A
B
4x
1.25x
1.25x
4x
1
2
3

**Fig. 8**

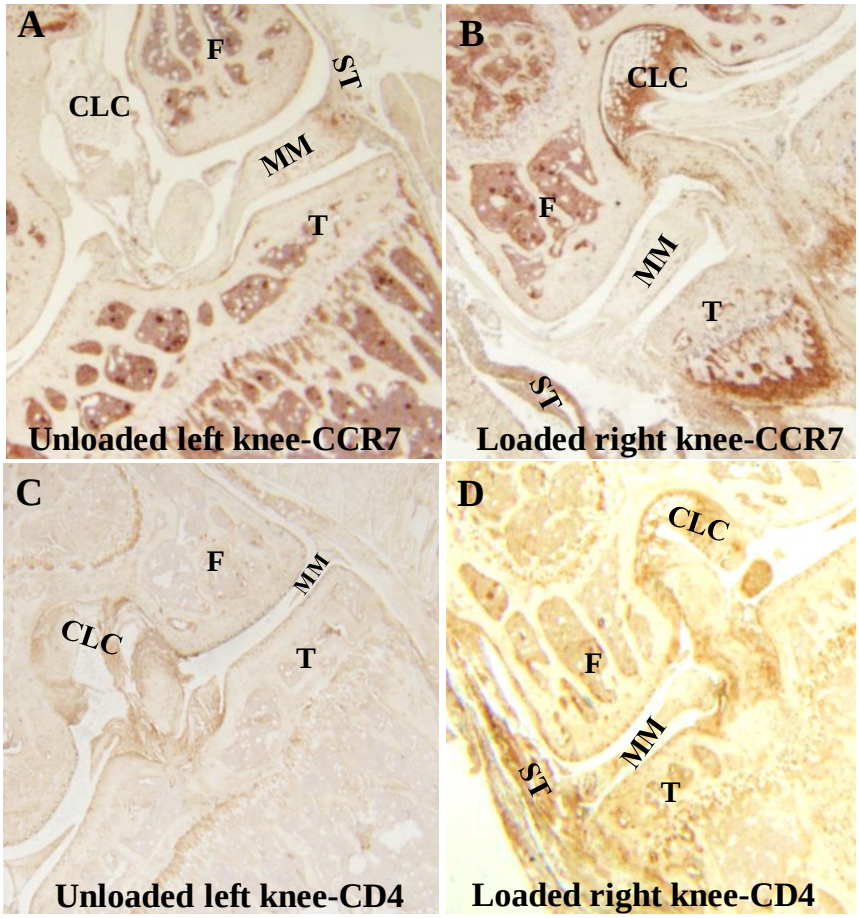

A
F
ST
CLC
MM
T
Unloaded left knee-CCR7
B
CLC
F
MM
T
ST
Loaded right knee-CCR7
C
F
MM
CLC
T
Unloaded left knee-CD4
D
CLC
F
MM
ST
T
Loaded right knee-CD4

**Fig. 9**

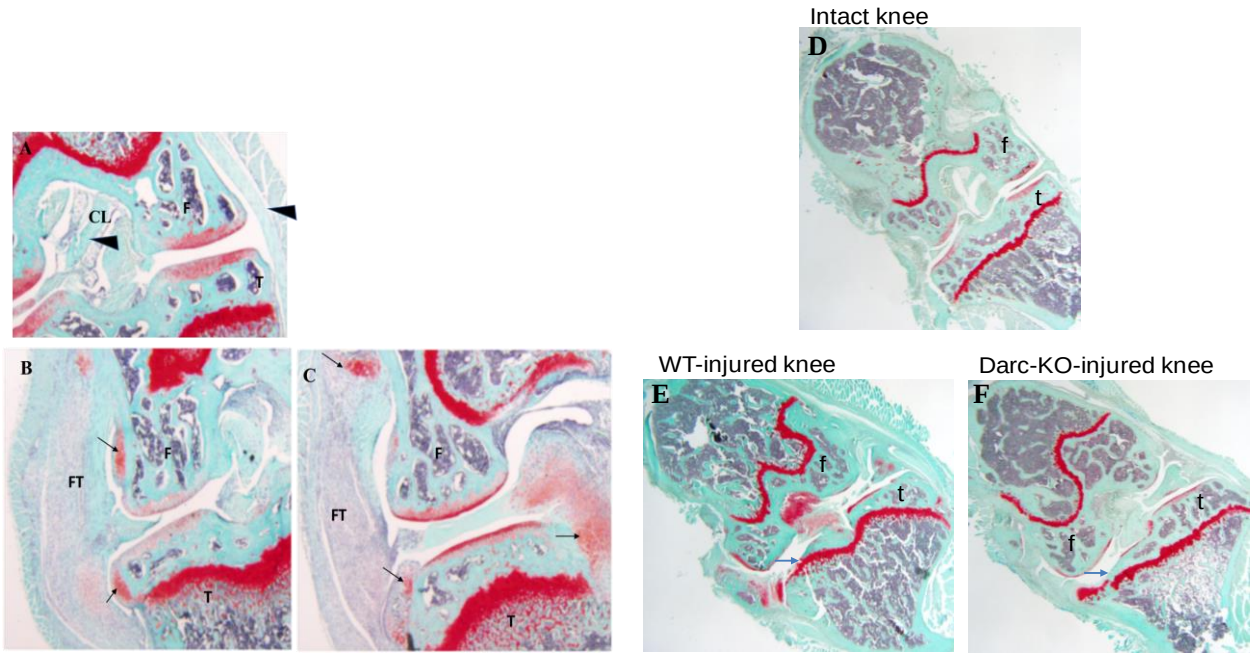
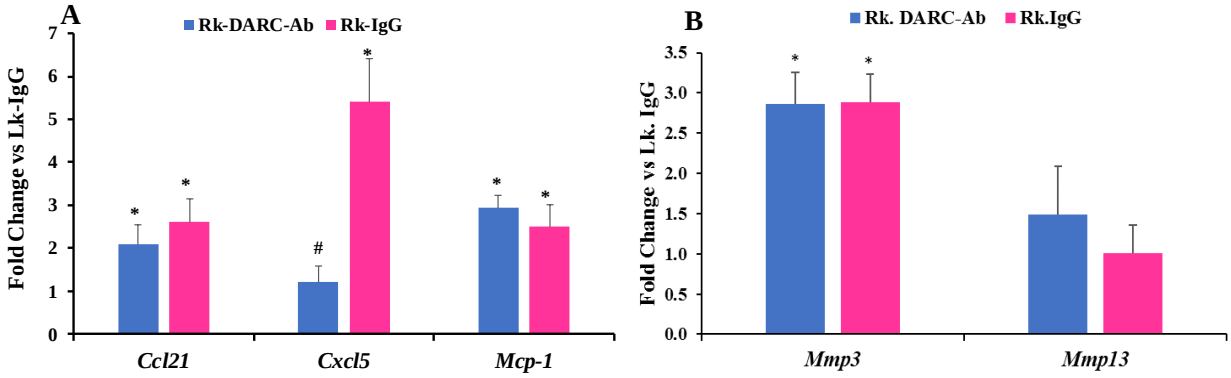

Intact knee
WT-injured knee
Darc-KO-injured knee
A
B
C
D
E
F
CL
F
T
FT
f
t

**Fig. 10**


A
Rk-DARC-Ab
Rk-IgG
Fold Change vs Lk-IgG
Ccl21
Cxcl5
Mcp-1
B
Rk. DARC-Ab
Rk.IgG
Fold Change vs Lk. IgG
Mmp3
Mmp13