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13. SUPPLEMENTARY NOTES

14. ABSTRACT Osteoarthritis is the most prevalent type of arthritis. It is characterized by progressive cartilage loss, synovial inflammation, with resultant joint pain that worsens over time.

While meniscectomy appears to be a significant risk factor for OA, researchers know little about the molecular pathways involved in the induction and development of osteoarthritis. In the present study, we proposed to investigate the role of the chemokines Ccl21 and Cxcl13 in inflammation caused by medial meniscectomy-knee destabilization (MMD) that leads to osteoarthritis. Therefore, we first evaluated the expression profile of these two chemokines in response to MMD in a rat knee injury model. mRNA levels for both chemokines were increased at 3 days and one week post-surgery. However, only *Ccl21* mRNA levels were significantly elevated in MMD knees compared to sham operated knees at 4 weeks post-surgery. This suggests that Cxcl13 is only involved in the first stages of osteoarthritis development, while Ccl21 is involved in both the early inflammation and the later stages of osteoarthritis development. Histological analysis revealed proteoglycan loss in the superficial zone of the articular cartilage that was obvious at 4 weeks post-surgery and in the calcified zone by 6 weeks post-surgery. These data confirm the involvement of CCL21 in cartilage catabolism. We are currently analyzing histological sections that were immune-stained to detect T and B cell markers, in order determine if there is a correlation between the expression of Cxcl13 and Ccl21, and the infiltration of inflammatory cells that occurs following knee surgery.

15. SUBJECT TERMS
Osteoarthritis, meniscectomy, inflammation

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Table of Contents

Page #

A. INTRODUCTION

Every healthy knee is supported and protected by a pair of menisci, C-shaped cartilage-like tissues located between the femur and tibia. The menisci serve many functions in the knee, including load bearing, shock absorption, and stability enhancement. In essence, the menisci serve as cushions that function to prevent joint cartilage degradation. In cases where the meniscus has been torn by injury, a common surgical treatment is to remove the damaged tissue. Surgical removal of all or part of a torn meniscus represents a significant risk factor for radiographic tibio-femoral OA. Inflammation in OA joints manifests with mononuclear cell infiltration observed in early and late stages of the disease. In terms of molecular pathways involved in the pathogenesis of the disease, a recent study (Loeser et al., 2012) reported an increased expression of Ccl21 in the destabilized joints of both young and old mice that went through medial meniscectomy destabilization (MMD). CCL21 was localized to chondrocytes, meniscal cells and the growth plate matrix. CCL21 is a ligand for CCR7 whose expression is also increased in the young MMD joints and in the old sham control joints. In humans, elevated CCL21 expression was observed in synovial tissue taken from OA patients, as compared to normal patients (Pickens et al., 2011). Data from previous studies suggested that both CD4+ and CD8+ are involved in OA development and that both CCL21 and CXCL13 may be involved in attracting these two T cell sup-populations to the injury site. Indeed, It has been reported that chemotaxis of Sezary Syndrome CCR7+ cells was significantly increased when CXCL13 was added to CCL21, thus suggesting that these two chemokines can also act synergistically to promote T cell migration (Zheng et al., 2005).

Objectives. In the present study, to investigate the role of CCL21 and CXCL13 on the recruitment of inflammatory cells to the injured knee and OA development after medial meniscectomy that causes knee destabilization (MMD), two specific aims were proposed;

In Specific Aim#1, the objective was to study the expression profile of CCL21 and CXCL13 induced by MMD and their role on the infiltration of the inflammatory cells.

In Specific Aim #2, the objective was to test the hypothesis that locale blockade of the function of CCL21 and/or CXCL13 will slow down OA development alter inflammation and stop cartilage degradation. To this end, the planned experimental approach was to compare the development of OA after surgery between the animals that received local injection of CCl21 or CXCL13 specific antibody or the antibodies against both chemokines and the mice that received IgG control.

B. BODY

Progress report during the First year of the funding period

1. **Specific aim#1.** Determine the expression profile of CCL21 and CXCL13 induced by MMD and their role on the infiltration of the inflammatory cells.

a. <u>Gene expression profile in response to joint instability caused by MMD</u>

a.1. Describe meniscectomy

Surgeries were performed on 10 week-old Sprague Dawley rats. All procedures were performed after approval from the Institutional Animal Care and Use Committee at the VA Loma Linda Health Care System. Under general anesthesia, the medial side of each knee joint

was shaved and the skin around the incision area was cleaned with 70% ethyl alcohol, Knee joint instability was induced surgically by a medial meniscectomy as previously described (Janusz et al., 2002; Bendele et al., 2001). The right knee was opened and the medial collateral ligament transected just below its attachment to the meniscus. Then, the meniscus was cut at its narrowest point taking care not to damage the tibia surface. The surgery results in knee destabilization (MMD). The left knee was sham operated through the same approach but without any ligament transaction and served as the contralateral control. Following surgery, tissue debris was removed by washing with sterile saline solution, and the incision then closed in two layers. The joint capsule and skin were sutured as previously described (Janusz et al., 2002).

Animals were sacrificed at different time points post-surgery. Tissues were collected by excising a region 3-4 mm above and below the middle of the knee joint, 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.

a.2. Gene expression profile at 1 and 3 days post-surgery

a.2.1. The expression levels of the two major inflammatory cytokines; *Il-6* and *Tnf*-alpha(*a*) as well as the two chemokines of interest in our study; *Ccl21* and *Cxcl13* was first evaluated at one and three days post-surgery using Real Time PCR as previously described (Edderkaoui et al., 2007). At one and three days post-surgery, the *Il-6* mRNA levels were increased by 7- and 4.3-fold, respectively, in response to MMD (**Fig. 1**). However, the level of *Tnfa* mRNA expression was slightly, but not significantly, affected by knee MMD **(Fig. 1**).

a.2.2. As for the two chemokines of interest to our study; *Ccl21* and *Cxcl13* mRNA levels were slightly increased, but not significantly increased in response to MMD at 1 day post-surgery (**Fig. 2A**). However, at 3 days post-surgery Ccl21 and Cxcl13 mRNA levels increased by 9- and 4- fold, respectively, in response to MMD (**Fig**. **2B**).

a.2.3. Two matrix metalloproteinases known for their catabolic effects on the extracellular matrix, MMP3 and 13, showed increases in mRNA levels of 17- and 4-fold, respectively, at 1 day post-surgery, and by 30- and 4-fold, respectively, at 3 days post-surgery, compared to sham operated knees (**Fig. 3**).

a.3. Gene expression profile at 1 and 4 weeks post-surgery

a.3.1. The expression levels of the two chemokines of interest to our study, *Ccl21* and *Cxcl1*3, were significantly increased in MMD knees at one week post-surgery compared to sham operated knees (**Fig. 4A**). However, the magnitude of increase was less than at 3 days post-surgery for both genes. At 4 weeks post-surgery, *Ccl21* mRNA levels were 3.5-fold greater than sham operated knees, while Cxcl13 mRNA levels were not significantly different than sham operated knees (**Fig. 4B**).

a.3.2. The mRNA levels of the two major Matrix metalloproteinases (MMPs), *Mmp3* and *Mmp13* was evaluated at 1 and 4 wks post-surgery. *Mmp3* mRNA levels were increased by 9- and 5-fold at 1 and 4 wks post-surgery, respectively, while *Mmp13* mRNA levels were increased by 3- and 2-fold compared to sham operated knees at 1 and 4 wks post-surgery (**Fig. 5**).

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

Intact knee joints were collected and muscle tissue was removed. The knees were fixed for three days in 10% buffered formalin, decalcified in EDTA for 30-34 days 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 Safranin-O/Fast green to assess general morphology and matrix proteoglycans. The samples were evaluated by two blinded observers to prevent bias. Microscopic examination of the articular cartilage was graded according to the Mankin's grading system (Mankin et al., 1971); this scoring system assesses structure (0 - 6 points), cellularity (0 - 3 points), matrix staining (0 - 4 points), and tidemark integrity (0 - 1points), and has a maximum of 14 points. The final score for each cartilage was based on the most severe histologic changes observed in multiple sections from each specimen.

Specimens from MMD knees showed irregularity of the superficial layer of the cartilage and loss of Safranin-O staining at the superficial zone at 4 weeks post-surgery (Fig. 6B) . At 6 weeks post-surgery, MMD knees showed reduced Safranin-O staining, not only at the superficial zone but also in the calcified cartilage layer (Fig. 6C). We also observed fibrillation and depletion of chondrocytes in the cartilage layer, and hyperplasia of chondrocytes, as well as cleft and irregular bony trabeculum between the junction of calcific cartilage and subchondral layers (**Fig.** 6**C**). In contrast, in the sham operated 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. 6A**). Moreover, significant differences in the histological OA scores were found between the sham operated group (1.06 ± 0.15) and the MMD group collected at 4 weeks (3.82 ± 1.3, $P<0.05$ vs sham) and 6 weeks post-surgery (8.5 ± 2.4, $P<0.05$ vs sham and vs 4 weeks post-surgery).

c. <u>Immunohistochemistry staining to assess the severity of inflammation and the infiltration of inflammatory cells in response to MMD.</u>

The severity of knee joint inflammation was reflected by an increase in the circumference of knee joints, observed at 1 and 3 days post-surgery in MMD knees, as compared to sham operated knees where no swelling was observed at these same times.

Before starting immunostaining, general observation of the sections stained with Hematoxylin-Eosin showed that joint spaces were filled with inflammatory secretions at 1 and 3 days post-surgery. We also observed hyperplasia and abundant mononuclear cells were present in the synovium.

For immunostaining studies, we have optimized the conditions for the use of CD4 and CD8 antibodies to assess the migration of T cells subsets in response to MMD, and CD20 antibodies to assess the level of B cell infiltration post-surgery.

C. KEY RESEARCH ACCOMPLISHMENTS DURING THE LAST 12 MONTHS OF FUNDING

We have made the following progress towards achieving the specific aims in this research project:

- We have evaluated the expression of the two major pro-inflammatory cytokines at 1 and 3 days post-surgery.
- We found a significant increase in the expression of *Il-6* both at 1 and 3 days post-surgery, while no significant difference was found in *Tnfa* mRNA expression levels in MMD-operated knees compared to sham operated knees.
- We have evaluated the change in the expression of the two chemokines of interest to our study, *Ccl21* and *Cxcl13*, as well as the two major effectors of the metabolism of articular cartilage, *Mmp3* and *Mmp13* during the 4 weeks post-surgery.
- The highest change in gene expression was found in the expression of *Mmp3*, a gene related to meniscus degeneration.
- In this study, we have shown that *Ccl21* mRNA levels were significantly increased from the first days post-surgery all the way to 4 weeks post-surgery suggesting a possible interaction between MMP3 and CCL21 during the development of OA.
- We have prepared different histological sections of the knees to compare the response of articular cartilage to knee instability caused by medial meniscectomy.
- Our evaluation of multiple histologic sections revealed the presence of irregularities and a loss of Safranin-O staining above the tidemark starting as early as 4 weeks post-surgery.
- Our evaluation of the sections prepared from knees collected at 6 weeks post-surgery revealed the presence of fibrillation, chondrocyte loss, and a reduction of Safranin staining, which extends into the radial zone. While the sections derived from the sham operated knees showed smooth superficial layer of cartilage, no disruption of surface integrity was observed. The cartilage matrix was well stained with Safranin-O. Preservation of Safranin-O and chondrocytes in calcified cartilage layer and smooth bony trabeculum was observed in subchondral layer.
- Our examination of the sections prepared from knees collected at 1 and 3 days post-surgery revealed inflammatory secretions at these time points. We have also observed hyperplasia and the presence of abundant mononuclear cells in the synovium.
- We have optimized the conditions for immunostaining with CD4 and CD8 antibodies to assess the migration of T cells subsets in response to MMD, and with CD20 antibodies to assess the level of B cell infiltration post-surgery.

D. CONCLUSION

Medial meniscectomy in rat knees induced the expression of multiple genes at different time points. Expression of Il-6, a major pro-inflammatory cytokines, and Mmp3 and Mmp13, known for their role in cartilage degradation, were all induced as early as one day post-surgery. Furthermore, *Mmp3* and *13* mRNA levels remained significantly elevated in MMD knees

compared to sham operated knees during the 4 weeks post-surgery period. On the other hand, we observed that mRNA levels for *Ccl21* and *Cxcl13* were increased at three days post-surgery and one week post-surgery. However, only Ccl21 mRNA levels were significantly elevated in MMD knees compared to sham operated knees at 4 weeks post-surgery. Interestingly, cartilage degradation manifested by loss of proteoglycan (observed as reduced Safranin-O staining) was observed as early as 4 weeks post-surgery. These data suggest that Cxcl13 is only involved in the first stages of osteoarthritis development, while Ccl21 is involved in both the early inflammatory response to knee injury, as well as the later stages where cartilage degeneration occurs with possible interactions between Ccl21 and MMPs that were found to be upregulated during the development of OA in our study.

We are in the process of analyzing the immune-stained sections derived from knees collected at 1 and 3 days post-surgery to determine if there is a correlation between the expression of Ccl21 and Cxcl13, and the infiltration of T and B cells into the joint space.

E. INVENTIONS, PATENTS AND LICENSES: Nothing to report

F. REPORTABLE OUTCOMES:

Expression of Ccl21 increased at three days post-surgery in MMD knees and remained elevated relative to sham operated controls during the four week post-surgery period. Increased expression of Mmp3 was observed as early as one day post-surgery in MMD knees and remained higher than the sham operated knee during the four weeks post-surgery period, suggesting a possible interaction between Ccl21 and MMP3.

G. OTHER ACHIEVEMENTS: Nothing to report

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I. FIGURE LEGENDS

Figure 1. mRNA expression levels of the two major inflammatory genes; *Il6* and *Tnfa* at the knee joints at (A) one day and (B) three days post-surgery. We collected tibia plate and condyles without meniscus from both sham and MM operated knees, data are presented as fold change compared to sham operated knees, n=6 and **P*<0.05 vs sham.

Figure 2. mRNA expression levels of the two inflammatory chemokines; *Ccl21* and *Cxcl13* at the knee joints at (A) one day and (B) three days post-surgery. We collected tibia plate and condyles without meniscus from both sham and MM operated knees, data are presented as fold change compared to sham operated knees, n=6-7 and **P*<0.05 vs sham.

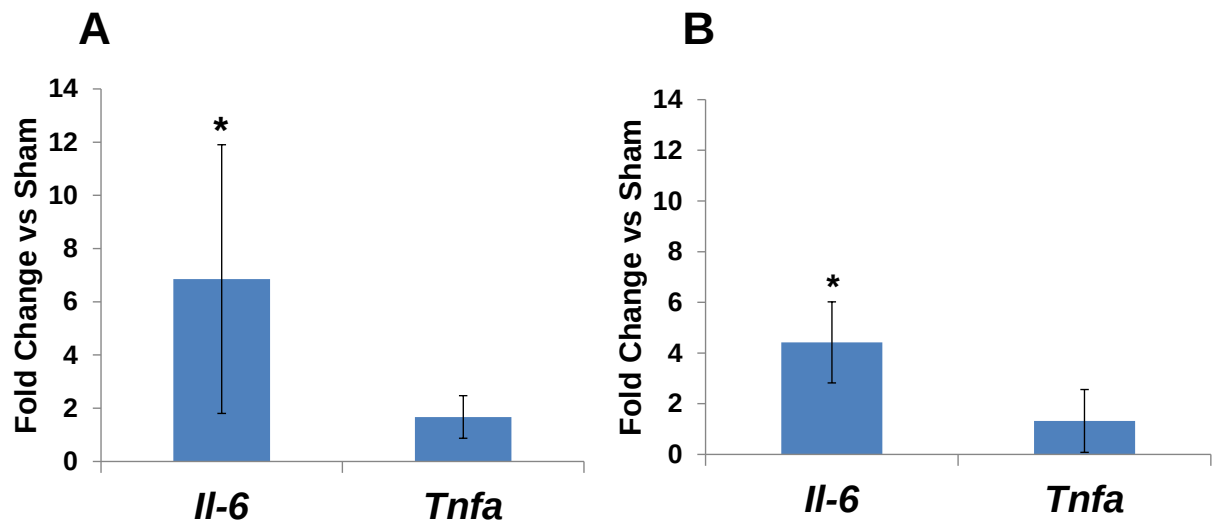
Figure 3. mRNA expression levels of the two major matrix metalloproteinases, Mmp3 and Mmp13 at the knee joints , at (A) one day and (B) three days post-surgery. Tibia plate and condyles, without menisci, were collected from both sham and MM operated knees-. Data are presented as fold change compared to sham operated knees, n=6-7 and **P*<0.05 vs sham.

Figure 4. mRNA expression levels of the two inflammatory chemokines; *Ccl21* and *Cxcl13* at the knee joints at (A) one week and (B) Four weeks post-surgery. We collected tibia plate and condyles without meniscus from both sham and MM operated knees, data are presented as fold change compared to sham operated knees, n=6-7 and **P*<0.05 vs sham.

Figure 5. mRNA expression levels of the two major effectors of the metabolism of articular cartilage, Mmp3 and Mmp13 at the knee joints at (A) one week and (B) Four weeks post-surgery. We collected tibia plate and condyles without meniscus from both sham and MM operated knees, data are presented as fold change compared to sham operated knees, n=6-7 and **P*<0.05 vs sham.

Figure 5. Histopathological evaluation of cartilage of the medial femoral condyles and tibia plate. (**A**) Sham operated knee showing smooth with no disruption of the AC surface integrity (black arrows), (**B**) medial condyle at 4 weeks post-surgery showing irregularity of the superficial layer of the cartilage and loss of Safranin-O staining at the superficial zone (black arrow)**,** (**C**) Medial condyle and tibia plate at 6 weeks post-surgery showing fibrillation, loss of Safranin-O staining and depletion of chondrocytes with irregular trabecular bony between the junction of calcified cartilage (black end arrows) Sections were stained with Safranin-O/Fast Green and observed using microscope objective 1.25 x. t. for tibia plate and f. for femur condyle.

Fig. 1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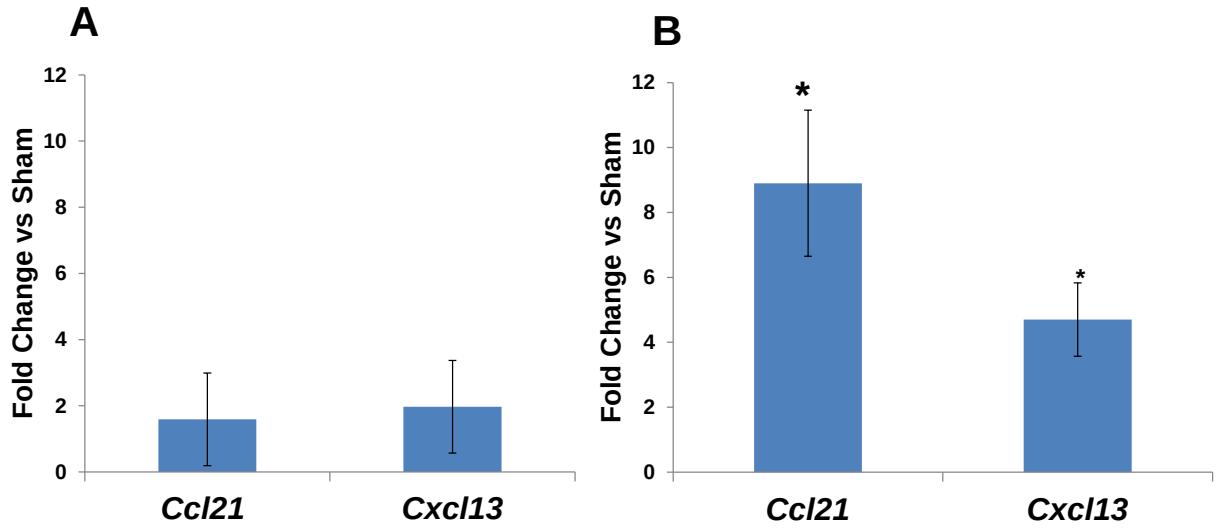
Il-6
Tnfa

Fig. 2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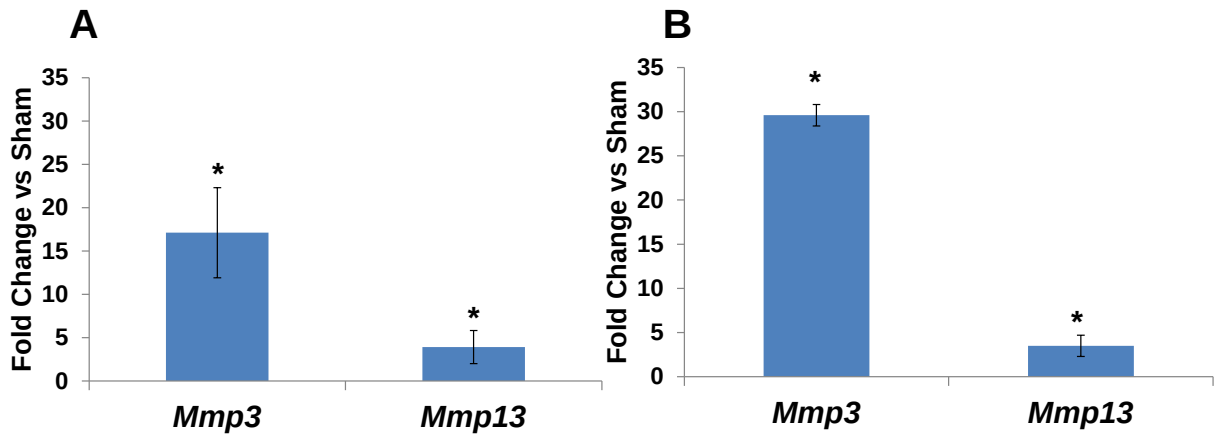
Ccl21
Cxcl13

Fig. 3



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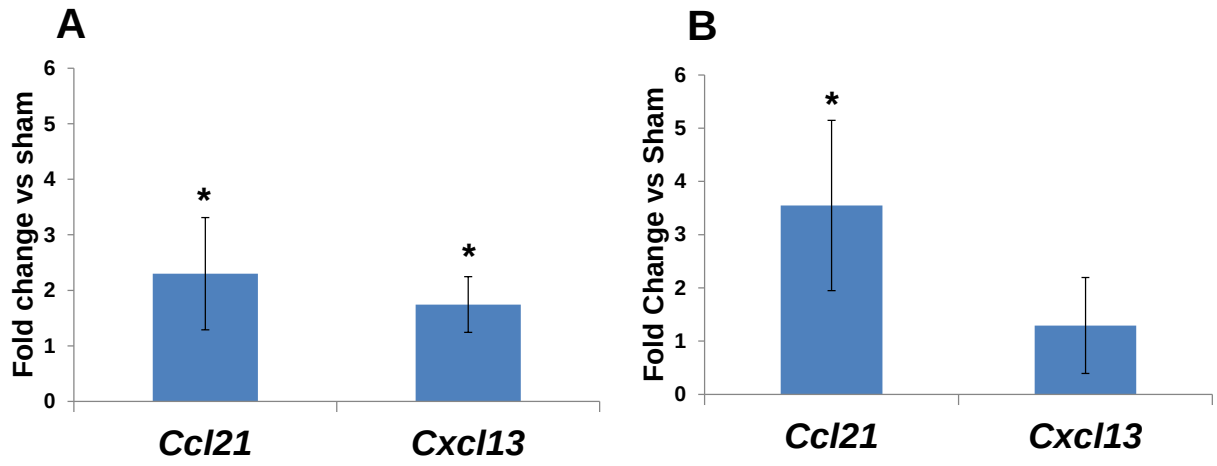

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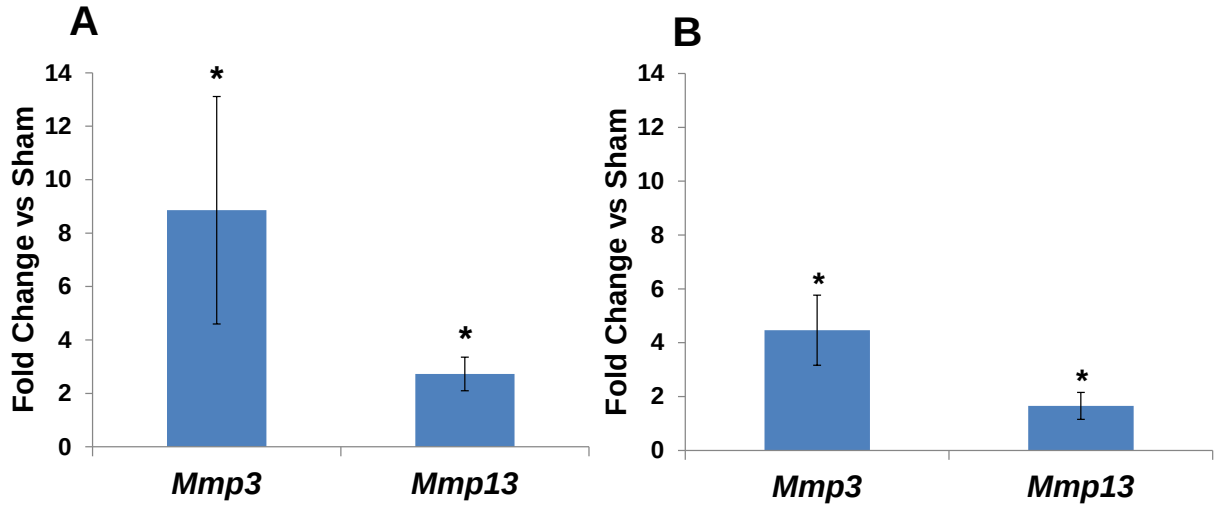

Fig. 6