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13. SUPPLEMENTARY NOTES

14. ABSTRACT. Osteoarthritis (OA) is characterized by progressive cartilage loss, with resultant joint pain that worsens over time. In the present study, we proposed to analyze the role of two chemokines, CCL21 and CXCL13, on OA development caused by partial medial meniscectomy (MMD). A novel hypothesis will be tested that local blockade of the function of CCL21 and CXCL13 at the knee joint will reduce inflammatory cells recruitment and prevent osteoarthritis development. Our first evaluation of the whole knee post-MMD was reported in our first annual report. In the last 12 months, we have assessed the infiltration of inflammatory cells post-MMD. A significant increase in the infiltration of CD4 and CD8 positive cells to the MMD knees was observed at 1 and 3 days post-surgery. MMD Knees treated with the CCL21 neutralizing antibody (Ab) caused a significant reduction in post-surgery inflammation. This was reflected by a reduction in the expression of the IL-6 and both T cell and B cells makers, which led to a significant reduction in the expression of matrix metalloproteinase 13 (MMP13) but no effect was observed in the expression of MMP3. However, the treatment with CXCL13-Ab caused an increase in post-surgery inflammation and in the expression of both MMP3 and MMP13. We are in the process of analyzing histology sections of MMD knees treated with CCL21-Ab for longer period to determine the role of CCl21 on OA development.

15. SUBJECT TERMS
Osteoarthritis, meniscectomy, inflammation

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A. INTRODUCTION

Post-traumatic osteoarthritis (PTOA) was diagnosed and recognized as a three year- disabling disease after injury occurrence. However, there are no treatments marketed for structural disease modification till now, so the need for novel preventive models and therapies is obvious. In this study, we have proposed to test a novel hypothesis that using small molecules to block locally the function of two chemokines (CCL21 and CXCL13) at the knee joint will reduce inflammation post-knee injury and therefore will prevent or slow down the pathogenesis of osteoarthritis. The successful confirmation of our hypothesis could lead to the development of therapies based on small molecules to prevent or treat the disease.

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) has 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 medial meniscectomy destabilized (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 sub-populations to the injury site. Indeed, it has been reported that chemotaxis of Sezary Syndrome CCR7+ cells were 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 control mice

B. BODY

Progress report during the period August 2016 – August 2017

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. 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 partial medial meniscectomy as previously described (Janusz et al., 2002; Bendele et al., 2001). The right knee was opened and the medial femoro-tibial 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 or meniscus tear 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).

a.2. Gene expression profile post-surgery

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., as previously described (Edderkaoui et al., 2007).

In our previous annual report, we have reported the data about the expression level of the two major inflammatory cytokines; Il-6 and Tnfalpha, as well as the expression of the two chemokines of interest in this study; The mRNA levels of Il-6, CCL21 and CXCL13 were significantly increased during the first 3 days post-knee surgery and MMD. In the last 12 months, to determine if the increase in the expression of Il-6 and the two chemokines of interest in this study are connected with inflammatory cell recruitment, the mRNA levels of the T cell and B cell markers were evaluated in synovial tissue at one day and from the whole knee at three days post surgery. Thus, specific primers were designed for CD20, which is an activated-glycosylated phosphoprotein expressed on the surface of all B-cells beginning at the pro-B phase and progressively increasing in concentration until maturity. It was used to evaluate the response of B cells to MMD, and the primers for CD3, CD4 and CD8 were designed to identify the response of different T cell subsets to knee MMD. The mRNA level was evaluated by Real Time quantitative PCR using cDNA generated from RNA isolated from both MMD knees and sham operated knees.

At one day post surgery, while the mRNA levels of CD4 and CD8 were increased by 7- and 4.6-fold in synovial tissue isolated from MMD knees compared to sham operated knees, respectively. The mRNA level of CD3 was slightly, but not significantly increased (1.7-fold, $P=0.09$) in MMD knees compared to sham operated knees (**Fig. 1**).

At three days post surgery (**Fig. 4**), Among T cell markers, the expression level of CD8 was the most affected at 3 days post-surgery, its mRNA level was increased by 10.6-fold in MMD knees compared to sham operated knees ($P<0.01$). CD4 mRNA level was increased by 8-fold ($P<0.05$), but the increase in the mRNA level of CD3 did not reach significance in MMD knees compared to sham operated knees. Furthermore, the expression of CD20 was increased by 5-fold ($P<0.05$) in MMD knees compared to sham operated knees. These data suggest that meniscus

injury induces the expression of CCL21 and CXCL13 (reported in our previous annual report), these later, create a chemotaxis and the recruitment of CD4+, CD8+, and CD20+ cells to the injured knees.

b. Immunohistochemistry staining to assess the infiltration of inflammatory cells in response to MMD.

To confirm our hypothesis that meniscus injury induces the expression of CCL21 and CXCL13 that create a chemotaxis and the recruitment of CD4+, CD8+, and CD20+ cells to the injured knees, we have performed immunostaining using specific antibodies for T cell and B cell markers.

The paraffin embedded sections were cleared and the sections were treated with 05% Triton for 10 minutes. Then, the sections were incubated with trypsin at room temperature for 1 minute. They were blocked for non-specific binding with 10% natural serum from the host of secondary antibody of each primary antibody, washed then incubated over-night with the primary antibody at 4oC. Sections were then incubated with secondary antibodies at 1:1000 for 1 hour at room temperature. Sections were counterstained with Mayer's Hematoxylin solution (IHCWorld).

After surgery, 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.

the operated knees showed an increase in the number of cells around the MMD knee joints at the medial side, and CD4+, CD8+ and CD20+ cells were obvious at 3 days post-surgery (**Fig. 2**). Though these cells were also present in the sham operated knees but their number was significantly lower than the MMD (**Fig. 2**)

2. 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.

To determine if the increased expression of CCL21 and CXCL13 is responsible of the recruitment of inflammatory cells and cartilage degradation, we have used CCL21 neutralizing antibody (Ab) to block the function of CCL21, and BCA1 neutralizing antibody to block the function of CXCL13.

Since the surgery was performed at the medial meniscus, the intra-articular injections of the antibodies were performed at the anteromedial knee between the condyle and the tibia plate, and under the patella, to treat the injured side of the knee.

10 µg rabbit polyclonal CCL21 antibody (Ab) was injected under the patella ligament in MMD operated kneed, using insulin syringes and 31G needles. The injections were performed one day post-surgery. Then, the effect of blocking the function of CCL21 on inflammation and cartilage degrading enzymes was assessed at three and five days post surgery.

b. Gene expression profile after blockade of CCL21 or CXCL13 function

b.1. Effect of CCL21 blockade on inflammation and cartilage degrading enzyme expression

We have first evaluated the change in the mRNA levels of the major pro-inflammatory cytokine; IL-6, the T and B cell markers, and two of the matrix metalloproteinase enzymes known for their role in cartilage degradation and OA. Indeed, the expression level of IL-6, CCl21 and CXCL13 was significantly reduced at three days post-surgery in the CCL21-Ab treated MMD-knees compared to control MMD-knees (**Fig. 3**). Furthermore, the mRNA levels of CD4 and CD8 as well as B cell marker CD20 were significantly reduced in the MMD knees treated with CCL21-Ab compared to the saline treated MMD knees (**Fig**. **4**). These data suggest a reduction in the infiltration of both T and B cells to the injured knee because of the alteration of CCL21 function. However, the reduction in inflammation did not affect MMP3 expression; since the mRNA level of MMP3 was similarly increased in the MMD knees treated with CCL21-Ab and the MMD knees treated with PBS (**Fig. 5**). In the other hand, the expression of MMP13 was significantly reduced in the MMD knees treated with CCL21-Ab compared to MMD knees treated with PBS (**Fig. 5)**.

b.2. Effect of CXCL13 blockade on inflammation and cartilage degrading enzyme expression 10 µg rabbit polyclonal BCA1 neutralizing antibody (Ab) for CXCL13 was injected under the patella ligament in MMD operated knees, using insulin syringes and 31G needles. The injections were performed at the same day of surgery. Then, the effect of CXCL13 neutralization on inflammation and cartilage degrading enzymes was assessed at three days post surgery. To our surprise, the expression levels of the two T cell markers as well as the B cell marker were significantly increased after treatment with CXCL13-antibody (Ab) in MMD knees compared to PBS treated MMD knees (**Fig. 6**). Then, we evaluated the expression of the two major matrix metalloproteinases, MMP3 and MMP13 (**Fig. 7**), a significant increase in the expression of both MMP3 and MMP13 after treatment of MMD knees with the neutralizing antibody for CXCL13 as compared with the MMD knees treated with PBS. We are in the process of analyzing the data from immunostaining to find out the reason of the increase in the mRNA levels of the two degrading enzymes, MMP3 and MMP13.

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 level of both B and T cell markers at 1 and 3 days post-surgery.
- We found a significant increase in the expression of both B cell marker; CD20, and two of the T cell markers, CD4 and CD8 both at 1 and 3 days post-surgery, but no significant difference in the expression of CD3, a marker of the another T cell sub-populations between MMD operated knees and the sham operated knees;
- We have performed immunostaining using markers of B and T cells to confirm the data from Real Time quantitative PCR
- An increase in the number of T cells, CD4 and CD8 positive cells was observed in the MMD knees compared to sham operated knees, at 3 days post surgery;
- The treatment with neutralizing antibodies against CCL21, reduced post-surgery inflammation and therefore reduced the expression of one of the major cartilage degrading enzymes; MMP13.
- The treatment with neutralizing antibodies against CCL21 that reduced post-surgery inflammation did not have any effect on the expression of MMP3.
- The treatment with neutralizing antibodies against CXCL13 increased post-surgery inflammation and therefore caused an increase in the expression of the two major cartilage degrading enzymes, MMP3 and MMP13.

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 cytokine, and Mmp3 and Mmp13, known for their role in cartilage degradation, were all induced as early as one day post-surgery. In the last 12 months, we have shown by gene expression evaluation and immunostaining, that MMD induced chemotaxis and the recruitment of CD4+ and CD8+ T cell sub-population as well as CD20+ B cells to the injured knees that was CCL21 dependent. Furthermore, we have shown that neutralizing the function of CCL21, improved the post-MMD surgery inflammation, and reduced the expression of one of the major cartilage degrading enzymes, the MMP13, but it did not affect the expression of MMP3.

We are in the process of analyzing more histology sections derived from MMD operated knees and sham operated knees treated with CCL21-Ab and/or CXCL13-Ab, at different time points to confirm the data from Real-Time quantitative PCR and to determine the effect of neutralizing the function of CCL21 and CXCL13 post-surgery on OA development.

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. Furthermore, our recent results showed that neutralizing the function of CCL21, improved the post-MMD surgery inflammation, and reduced the expression of one of the major cartilage degrading enzymes, the MMP13, but it did not affect the expression of MMP3.

G. OTHER ACHIEVEMENTS: Nothing to report

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I. FIGURE LEGENDS

Figure 1. mRNA expression levels of two T cell markers increased at 1 day post knee surgery. We collected knees from both sham and MMD operated knees, data are presented as fold change compared to sham operated knees, n=4 and **P*<0.05 vs sham.

Figure 2. Representative images from immunostaining of MMD (right panels) and sham (left panel) operated knees. Antibodies for CD4 (**A**), CD8 (**B**) and CD20 (**C**) were used. M. meniscus, ST. synovial tissue. Arrows show the positive cells.

Figure 3. mRNA levels of the major pro-inflammatory cytokine IL-6 and the chemokines CCL21 and CXCL13 were reduced by the alteration CCL21 function at 3 days posts knee surgery. Data are expressed as fold change vs sham operated and PBS treated knees. n=4, *P<0.05 vs Sham saline injected, #P<0.05 MMD. PBS vs MMD. CCL21-Ab treated.

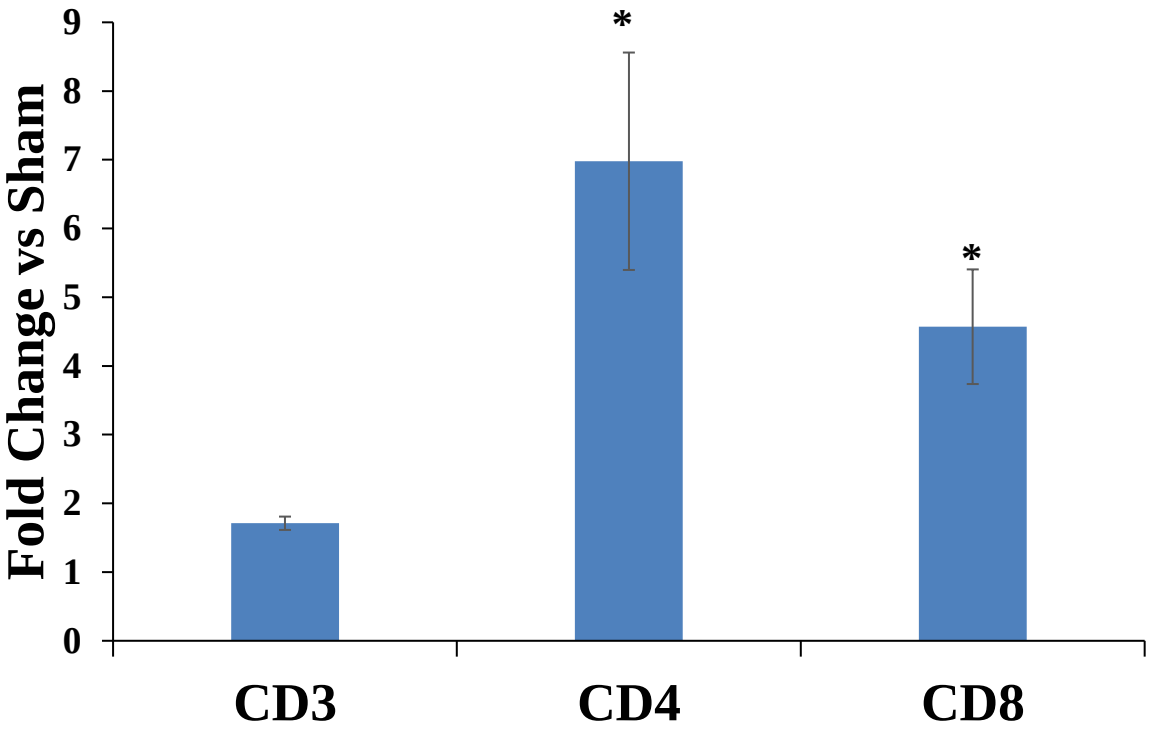
Figure 4. mRNA levels of two T cell markers and one B cell markers were reduced by the alteration CCL21 function at 3 days posts post knee surgery. Data are expressed as fold change vs sham operated and PBS treated knees. n=4, *P<0.05 vs Sham saline injected, #P<0.05 MMD. PBS vs MMD. CCL21-Ab treated.

Fig. 5. No change in the mRNA level of matrix metalloproteinase enzyme MMP3 but the mRNA level of MMP13 was significantly reduced by alteration of CCL21 function at 3 days posts post knee surgery. Data are expressed as fold change vs sham operated and PBS treated knees. n=4, *P<0.05 vs Sham saline injected, #P<0.05 MMD. PBS vs MMD. CCL21-Ab treated.

Fig. 6. mRNA levels of two T cell markers and one B cell markers were significantly increased by the alteration of CxCL13 function at 3 days posts post knee surgery. Data are expressed as fold change vs sham operated and PBS treated knees, n=4, *P<0.05 vs Sham saline injected, #P<0.05 MMD. PBS vs MMD. CxCL13-Ab treated.

Fig. 7. A significant increase in the mRNA level of the two major matrix metalloproteinase enzymes; MMP3 and MMP13 after treatment with CXCL13 antibody (Ab), at 3 days posts post knee surgery. Data are expressed as fold change vs sham operated and PBS treated knees. n=4, *P<0.05 vs Sham saline injected, #P<0.05 MMD. PBS vs MMD. CxCL13-Ab treated

Fig. 1.



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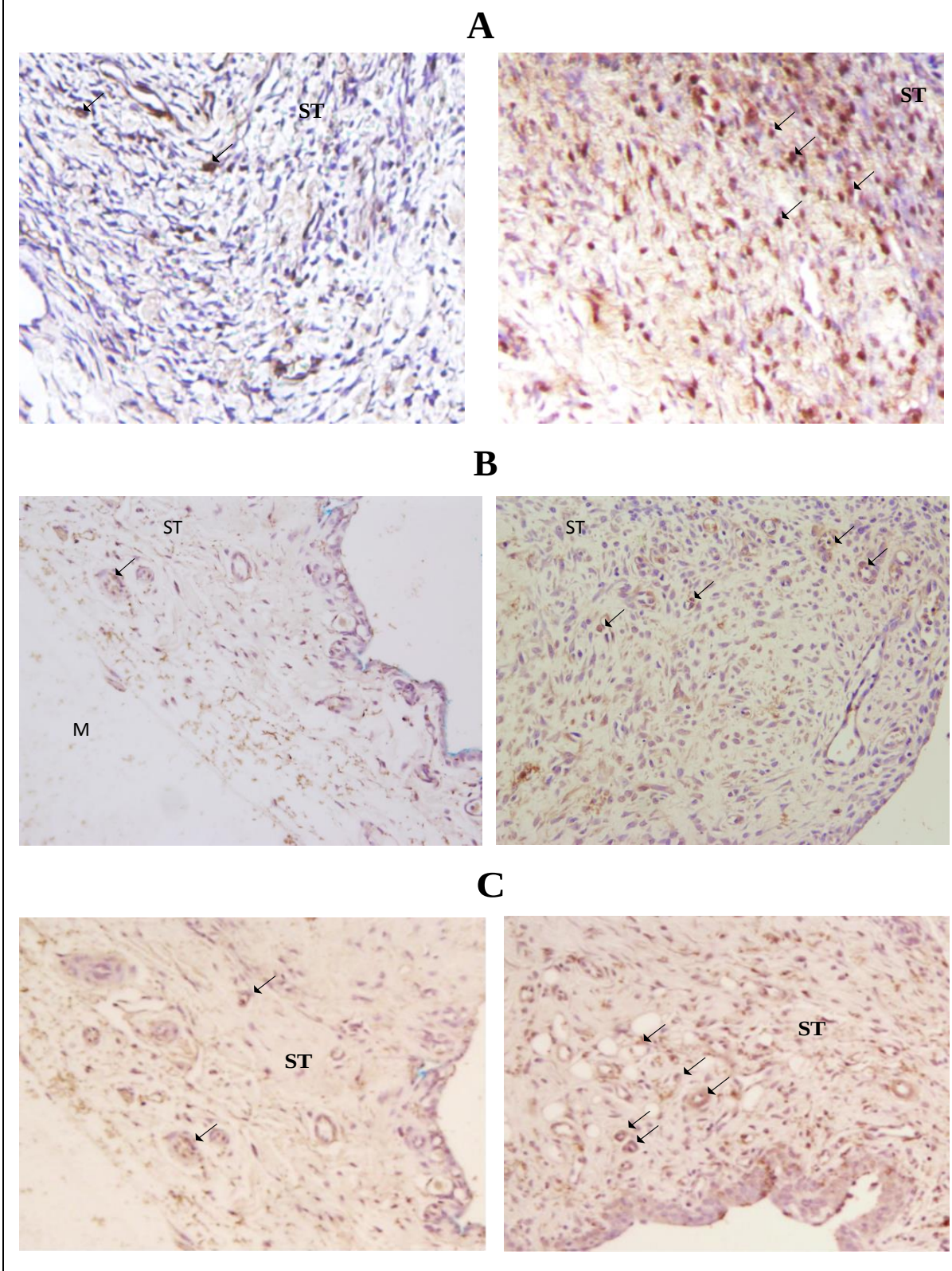

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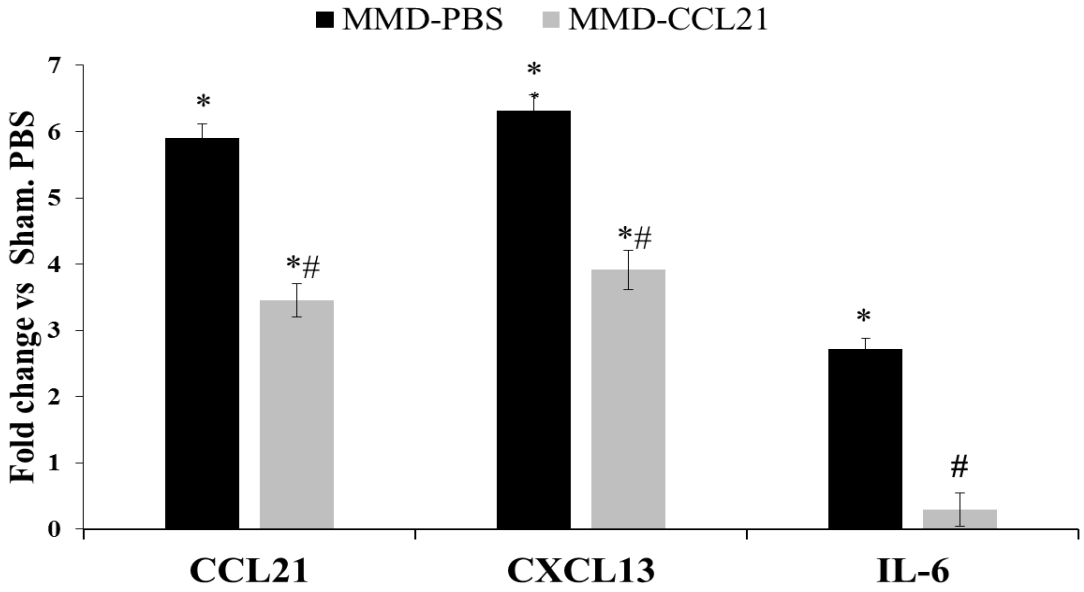
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CCL21
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IL-6

Fig. 4

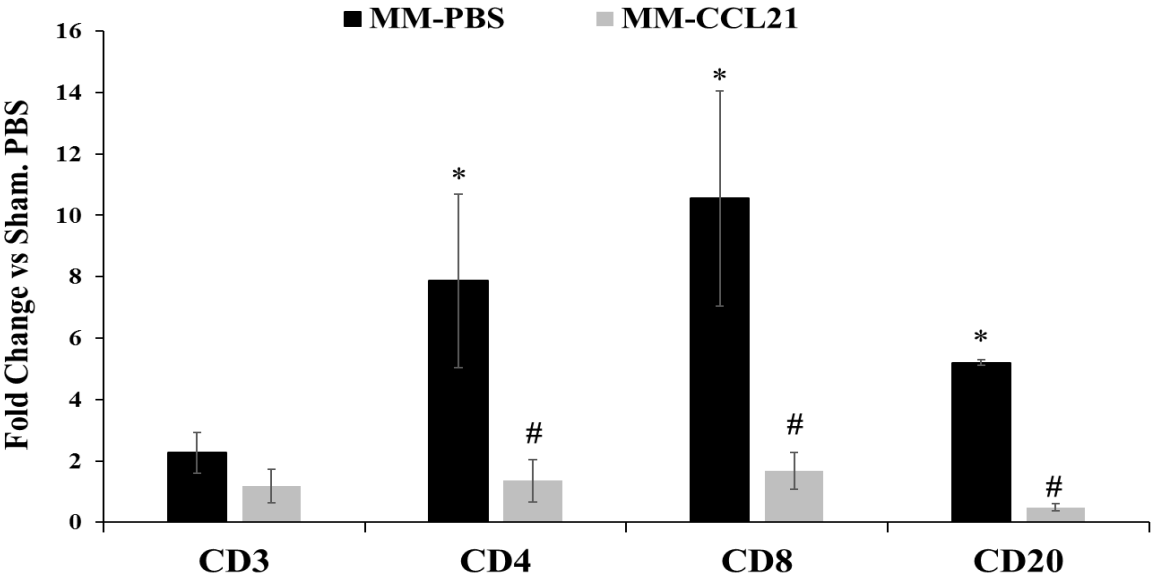
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Fig. 5.

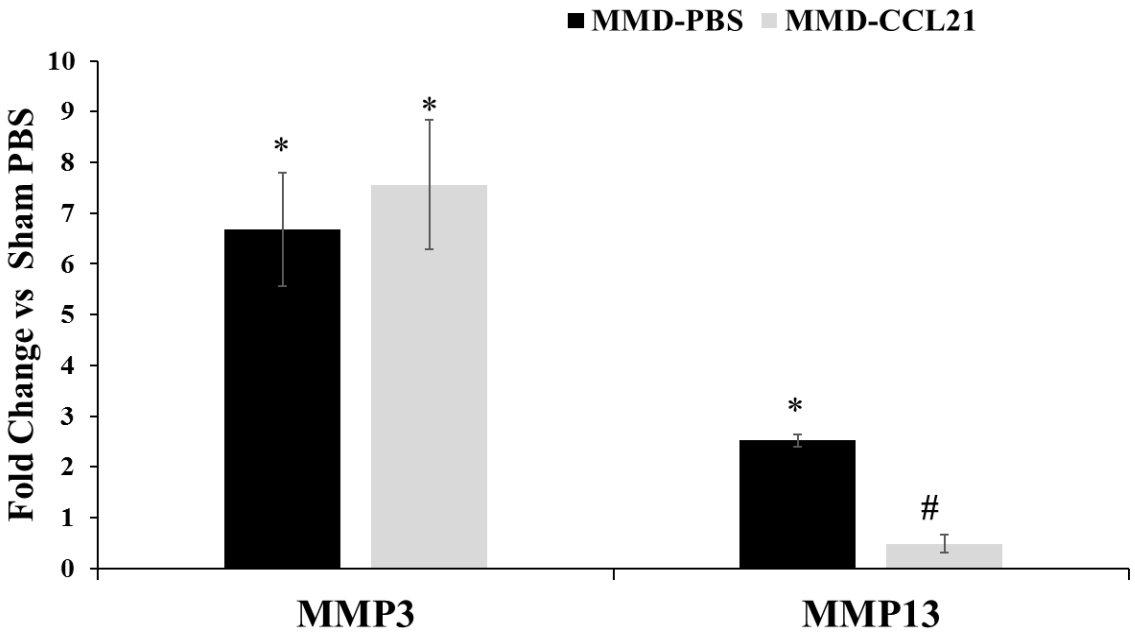
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Fig. 6

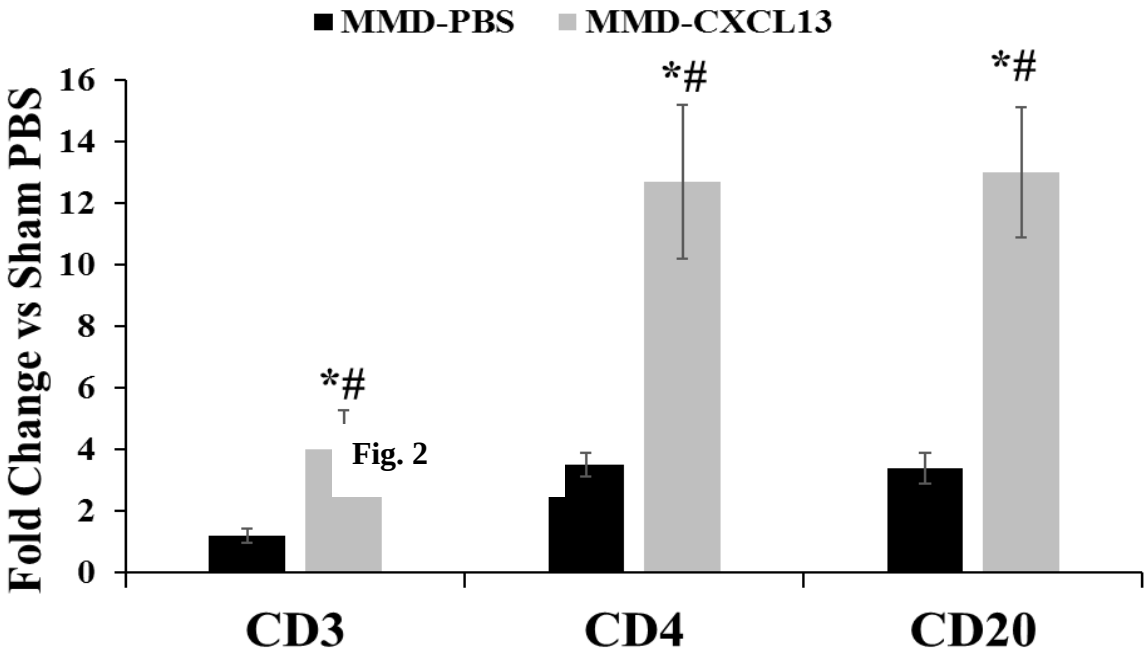
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Fig. 2
CD3
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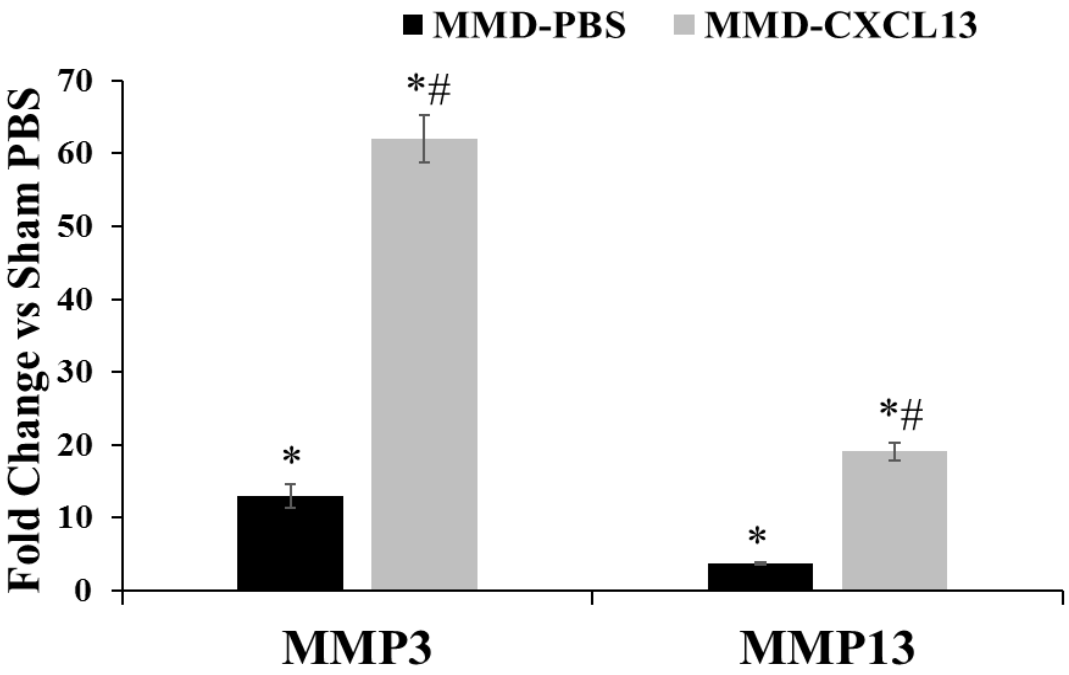
Fig. 7.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