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Award Number: W81XWH-13-1-0228

TITLE: Development of an Autologous Macrophage-based Adoptive Gene Transfer Strategy to Treat Posttraumatic Osteoarthritis (PTOA) and Osteoarithritis (OA)

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REPORT DATE: September 2014

TYPE OF REPORT: Annual

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**
September 2014	Annual	1 Sep 2013 - 31 Aug 2014

4. TITLE AND SUBTITLE
Development of an Autologous Macrophage- Based Adoptive Gene Transfer Strategy to Treat Posttraumatic Osteoarthritis (PTOA) and Osteoarithritis (OA)

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-13-1-0228

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Loma Linda University

Loma Linda, CA 92350

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
OA is the most common degenerative joint disease, and ~12% of all OA are resulted from an acute trauma to the joint and are referred to as PTOA. There is no cure for PTOA or OA. This Discovery Award project seeks to obtain proof-of-concept type of evidence for the feasibility of and efficacy for an innovative autologous macrophage-based anti-catabolic and pro-chondrogenic combination adoptive gene therapy for treatment of PTOA. The rationale for the use of macrophages as the cell vehicle for targeted delivery and confined expression of the transgene(s) is based on definitive evidence that a) PTOA development is associated with both acute and chronic inflammation of the synovium; and b) synovial inflammation triggers massive infiltration of activated macrophages. The idea of the combination macrophage-based adoptive gene therapy with both an anti-catabolic gene (IL-1ra or IL-1β shRNA) and a pro-chondrogenic gene (TGFβ3) is based on the assumption that comprehensive treatment of a disease with complex pathophysiology, such as PTOA, will require concerted treatments at multiple phases of the diseases. The proposed study will test two hypotheses: 1) *the autologous macrophage-based adoptive gene transfer strategy can effectively deliver and confine expression of an anti-catabolic gene (IL-1ra or IL-1β shRNA) along with a chondrogenic gene (TGFβ3) in the inflamed areas within the synovium of the PTOA joint*; and 2) *the IL-1ra (or IL-1β shRNA) and TGFβ3 combination autologous macrophage-based adoptive gene transfer strategy will reduce PTOA symptoms and promote articular cartilage regeneration in a mouse PTOA model*. Aim 1 will show that: 1) intra-articular injection of donor macrophages will lead to confined and long-term recruitment of donor cells at the inflamed surface of the injured articular cartilage; 2) C57BL/6J macrophages can be effectively transfected by lentiviral vectors to express large amounts of transgene; and 3) intra-articular injection of genetically modified syngenic mouse macrophages into the PTOA synovium will result in confined and sustained production of large amounts of the transgenes (i.e., IL-1ra and TGFβ3) at and around the surface of the inflamed articular cartilage. Aim 2 will test the dose- and time-dependent effects of the macrophage-based IL-1ra/TGFβ3 combination adoptive gene therapy on the suppression the inflammation-induced degradation of articular cartilage (and/or erosion of endochondral bone) and on the stimulation of the TGFβ3-mediated chondrogenesis to repair and regenerate the damaged articular cartilage, respectively. This work will be performed in a mouse intra-articular tibial fracture PTOA model (with C57BL/6J mice).

15. SUBJECT TERMS: nothing 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
U	U	U	UU	**15**	**19b. TELEPHONE NUMBER** *(include area code)*

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I. Introduction:

Osteoarthritis (OA) is the most common degenerative joint disease, and ~12% of all OA are resulted from an acute trauma to the joint and are referred to as PTOA [1]. It is estimated that the total cost of OA cases is close to $100 billion, of which nearly 50% is from lost earnings [2, 3]. Military personnel have a significantly greater incidence of PTOA than the general population of the same age range [4], which is likely due to the intense physical demands of military-related activities or combat-related traumatic joint injuries. Either direct joint damage or limb amputation result in increasing loads on contralateral joint surfaces, gradually leads to the development of PTOA. There is no cure for PTOA or OA. Treatment options vary, including physical therapy, lifestyle changes, orthopedic bracing, and medications. Joint replacement surgery may also be required in eroding forms of arthritis. Medications can help reduce inflammation in the joint to reduce pain, and slow the progress of joint damage [5]. This project seeks to obtain proof-of-concept type of evidence for the feasibility of and efficacy for an innovative autologous macrophage-based anti-catabolic and pro-chondrogenic combination adoptive gene therapy for treatment of PTOA, and tests two specific hypotheses: 1) *the autologous macrophage-based adoptive gene transfer strategy can effectively deliver and confine expression of an anti-catabolic gene (IL-1ra or IL-1β shRNA) along with a chondrogenic gene (TGFβ3) in the inflamed areas within the synovium of the PTOA joint*; and 2) *the IL-1ra (or IL-1β shRNA) and TGFβ3 combination autologous macrophage-based adoptive gene transfer strategy will reduce PTOA symptoms and promote articular cartilage regeneration in a mouse PTOA model*. If successful, this therapy will provide an innovative, safe, cost-efficient and non-invasive alternative for treatment of PTOA and other forms of arthritis. Thus, this proposal has very high clinical significance to the military, veterans, and civilian populations.

II. Keywords:

Osteoarthritis; Post-traumatic; IL1ra; TGFbeta3; Transduction, Adoptive therapy, Macrophages, Chondrocytes; Articular Cartilage; Regeneration; Mice

III. Overall Project Summary:

The primary objective of this 18-month proposal is to obtain proof-of-concept evidence for the prevention and treatment using this novel macrophage-based adoptive gene transfer approach. This project has two Specific Aims:

Aim 1. To demonstrate that the macrophage-based adoptive gene transfer strategy can effectively deliver and yield confined, sustained expression of transgenes in the inflamed synovia of the PTOA.
Aim 2. To demonstrate that the macrophage-based IL-1ra (or IL-1β shRNA) and TGFβ3 combination adoptive gene therapy can effectively treat and prevent PTOA in a mouse PTOA model.

These two Aims will be accomplished through the following four Technical Objectives (milestones):

Regulatory review and approval and recruitment of the research technician – Month 0 to Month 3.

Milestone 1 (Technical Objective 1). *To demonstration that the macrophage-based adoptive gene transfer strategy can effectively deliver and yield confined expression of transgenes in the inflamed synovium of the PTOA* (Aim 1).

Specific Task 1 is to establish the closed intra-articular tibial plateau fracture PTOA model in the knee of C57BL/6J mice for use in subsequent work – Month 3 to Month 6.

Specific Task 2 is to demonstrate that the injected donor GFP-expressing M2 macrophages are recruited to and retained in the inflamed articular cartilage surface in the synovium of the PTOA knee joint for substantial period of time after intra-articular injection (Aim 1a) – Month 6 to Month 12.

Milestone 2 (Technical Objective 2). *To demonstrate that primary mouse macrophages can be effectively transduced with lentiviral vectors and express substantial amounts of the transgenes (i.e., IL-1ra or TGFβ3).*

Specific Task 1 is to construct the required lenti-viral based vectors that can effectively express murine IL-1ra, TGFβ3, or GFP marker genes - Month 3 to Month 5.

Specific Task 2 is to confirm that the isolated mouse macrophages can be effectively transduced by the lentiviral vectors expressing IL-1ra, TGFβ3, or GFP *ex vivo*, and to confirm that the transduced cells express substantial amounts of the transgene (Aim 1b) – Month 5 to Month 10.

Milestone 3 (Technical Objective 3). *To develop the macrophage-based adoptive therapy expressing IL-1ra and/or TGFβ3 can be used to treat established PTOA* (Aim2a).

Specific Task is to test whether direct injection of the optimal dosage (determined in Specific Task 1 of Milestone 1) of genetically modified macrophages expressing IL-1ra and/or TGFβ3 into the synovium of the injured joint with established PTOA would improve the PTOA symptoms – Month 10 to Month 18.

Milestone 4 (Technical Objective 4). *To demonstrate that the macrophage-based IL-1ra and TGFβ3 combination adoptive gene therapy can effectively prevent development* of *PTOA* (Aim 2b).

Specific Task is to determine whether the adoptive therapy can be used to prevent development of PTOA – Month 10 to Month 18.

This annual progress report will summarize our progress from September 1 2013 to July 15 2014 and describe our progress towards Milestone 1 and 2.

a. Regulatory review and approval and recruitment of the research technician – From September 1, 2013 to December 15, 2013, most of our efforts in this project were spent on getting approval for our proposed work from all necessary committees, including our institutional IACUC, IBC, and ACURO. It has also taken us several months to successfully recruit Dr. David Xu to work as a half-time research staff on this project.

b. Progress toward Milestone 1, Subtask 1: To establish the closed intra-articular tibial plateau fracture PTOA model in C57BL/6J mice.

PTOA is frequently caused by the sudden application of mechanical force (impact) to the articular surface. Greater local tissue damage, such as chondrocyte death and matrix disruption, is the common consequence of the high-energy impact. Our first task was to establish a mouse PTOA model, because our laboratory did not have a valid PTOA model at the time of this investigation. Accordingly, we chose a previously published procedure [6] to create the intra-articular PTOA fracture model in the knee of C57BL/6J (B6) mice at 8-10 weeks. This method generates the tibial plateau fractures resembling to the clinical situations seen in PTOA. Very briefly, the right knee of B6 mice is placed onto an Instron mechanical tester (left panel of Fig.1), capable of delivering a pre-determined compression load. A custom-made stainless steel wedge shaped indenter (Right panel of Fig.1) was mounted to the testing system to apply a fixed compressive preload to the joint.

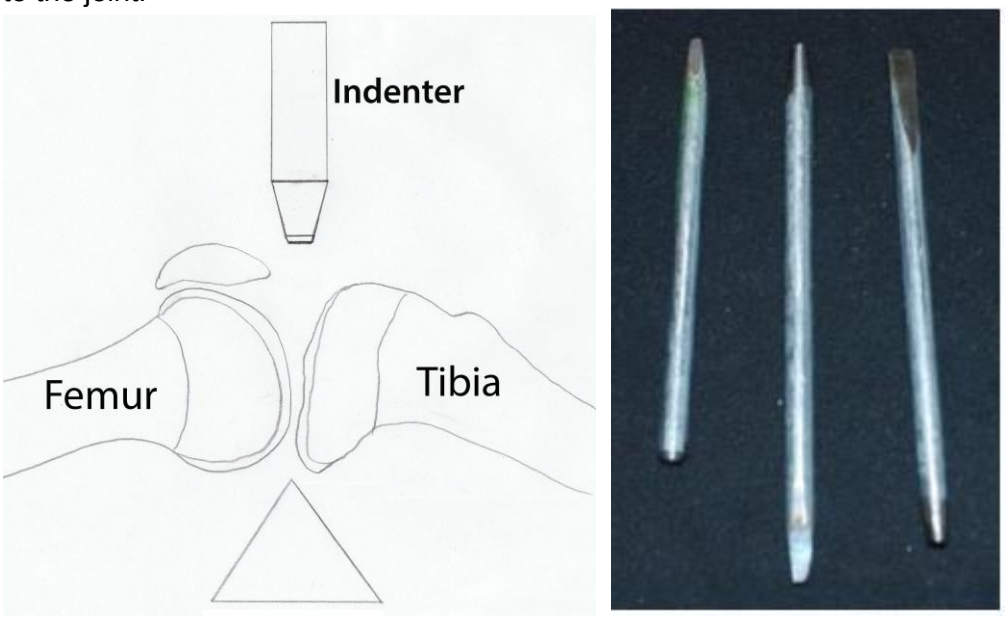

Figure 1: The intra-articular tibial plateau fracture model. This scheme cartoon (left panel) was modified from Furman [6]. In this model, the mouse knee joint is bended and placed on a supporting bar. An indenter is placed on the tibial plateau and a mechanical force is applied through an indenter using an Instron mechanical tester to create partial intra-articular fractures. The indenter was custom-made from steel rod with different tip width (right panel).

To obtain site-specific articular fracture, we used the instron tester equipped with the software, WaveMatrix (version 1.3) to manage the degree of damage of fractures on the tibial plateau (Fig. 2). To specifically observe the knee injury after this mechanical insult, one PTOA knee was dissected at day1 post injury. As shown in Fig. 3, the injury versus contra-lateral knee displayed intra-articular bleeding, a classical feature of clinical PTOA.

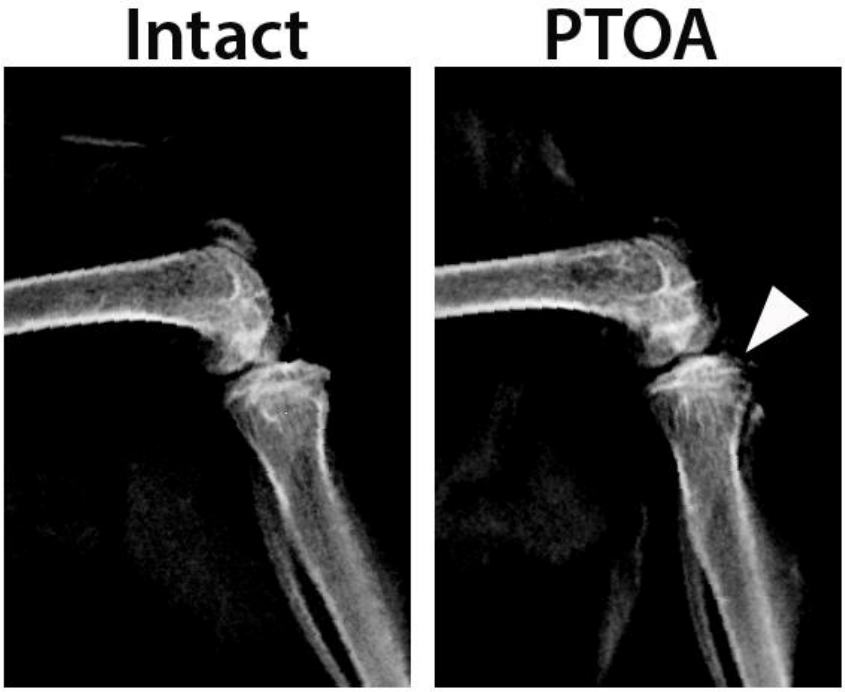

Figure 2: The comparison of the injured (right panel) versus contra-lateral control (left panel) knees. The damaged (fracture) site is indicated by the arrowhead.

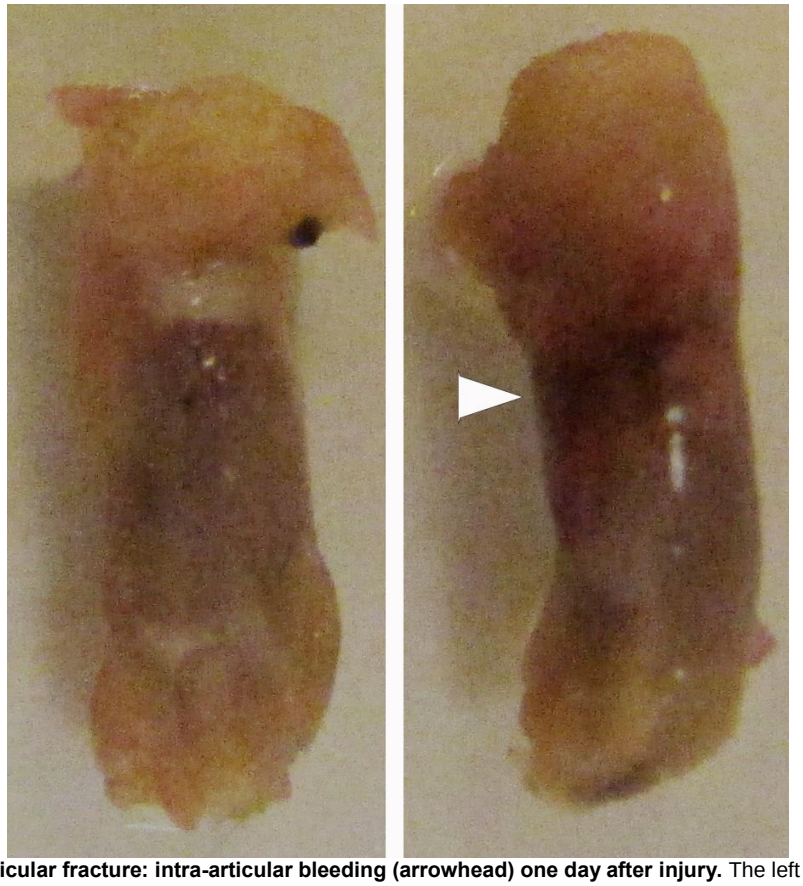

Figure 3. Evidence of closed articular fracture: intra-articular bleeding (arrowhead) one day after injury. The left panel is the knee from the

contra-lateral control.

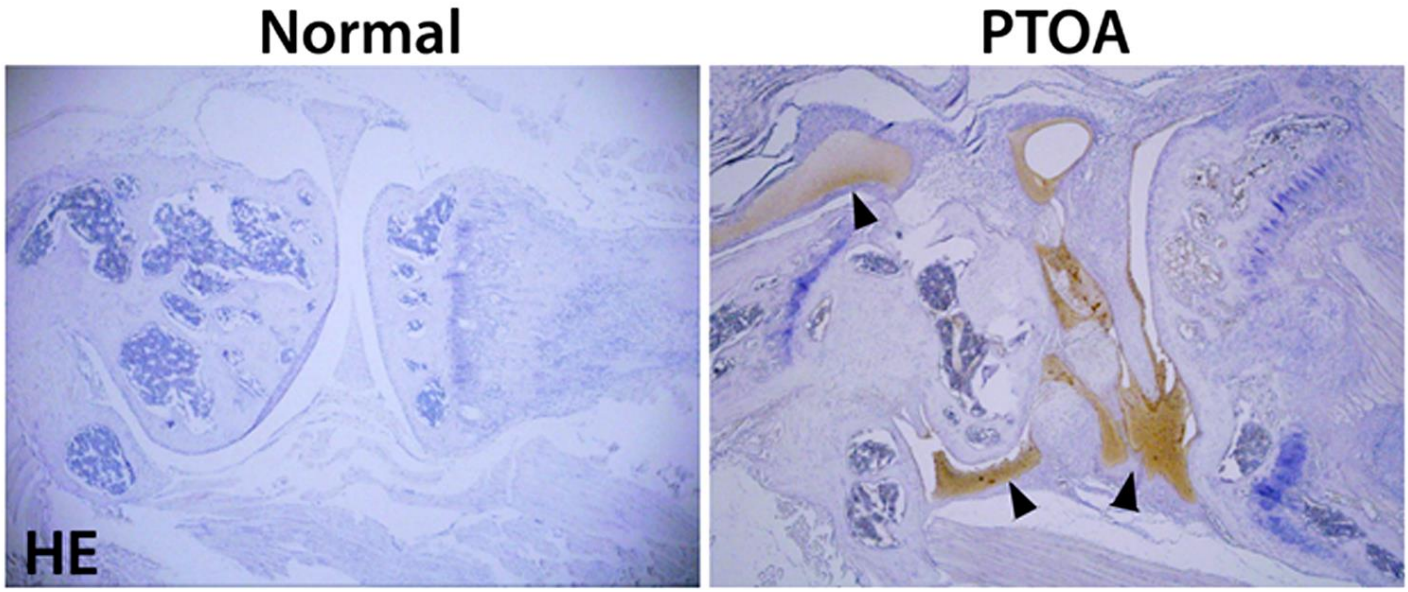

Figure 4: Inflamed synovial fluid (arrowheads) in injured (right) versus contra-lateral (left) knees 10 days post fracture. The paraffin-embedded sections were stained by H&E.

PTOA is caused by direct or indirect impact, leading to the immediate damage of joint structures such as meniscus and ligaments. After injury, our mouse PTOA joint clearly showed a sign of synovial inflammation which is composed of a mix of immune cells, dead chondrocytes, proteinases/cytokine, and degraded cartilage (Fig. 4).

To confirm the development of PTOA in our mouse model, we scanned the injured knee and the contralateral intact knees of mice at 12 weeks post surgery by µCT. Fig. **5** shows the three-dimensional reconstruction of the anterolateral view (left), lateral view (middle), and medial view (right) of the right PTOA knee joint and the contralateral left intact knee joint of a representative mouse. Clear evidence for intra-articular fractures, existence of substantial subchondral bone erosion at the surface of articular plate, and formation of bone spurs (small growths called osteophytes) on the edges is seen in the PTOA joint but not on the intact contralateral knee joint. This provides addition strong evidence that PTOA was developed at the injured knee joints.

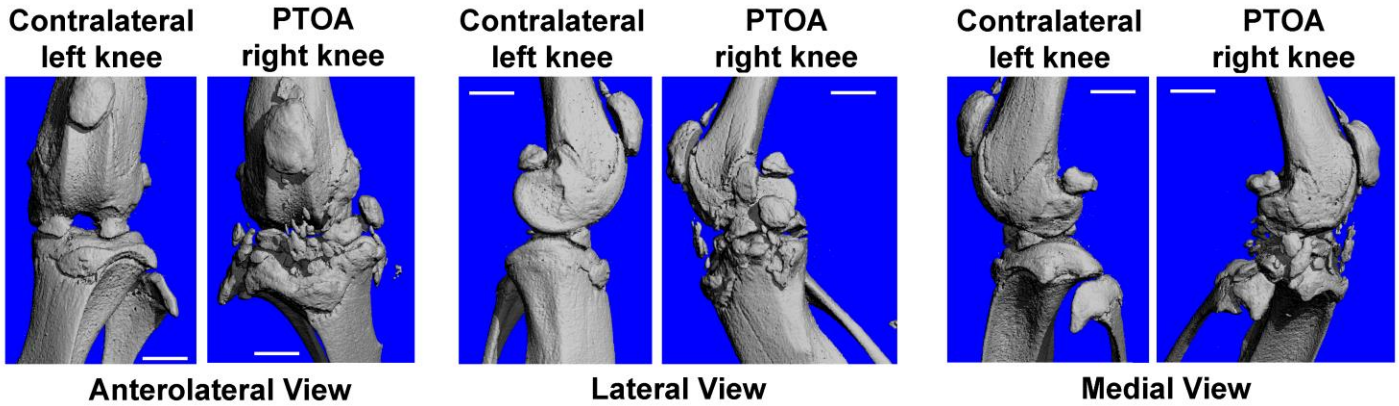

Figure 5. The anterolateral view (left), lateral view (middle), and medial view (right) of micro-CT three dimensional reconstruction of the fractured right knee joint and the contralateral intact left knee joint of a representative mouse at 12 weeks post-fracture. Bars = 1 mm.

Collectively, the data from our preliminary studies are consistent with current knowledge about the PTOA progression [5]. Consequently, we have developed a mouse model of PTOA.

Recent study of Anderson et al [7] suggests that the development of PTOA can be segregated into two major phases: 1) inflammation and cell death during the early stage and 2) remodeling responses during the late stage. To effectively prevent and treat PTOA, more detailed understanding of the disease progression at both phases is critical and necessary. In order to appropriately assess structural, cellular, and/or molecular

changes at the joint after development of PTOA, we have also developed histological methods to monitor these changes, since histology is the common tool to evaluate the cellular and structure changes during the development of PTOA.

To study the pathophysiology of PTOA progression, we evaluated the injury versus contra-lateral joints collected at 10, 28, 56, 84, and 112 days post-fracture. The paraffin-embedded thin sections were processed for Safranin-O staining specific for the articular cartilage. As shown in **Fig. 6**, degradation of articular cartilage was observed at 10 days post-fracture in the injured but not the intact contralateral intact knee by the Safranin-Orange staining. The red staining became weaker and thinner, which is a well-known hallmark of the early development of PTOA.

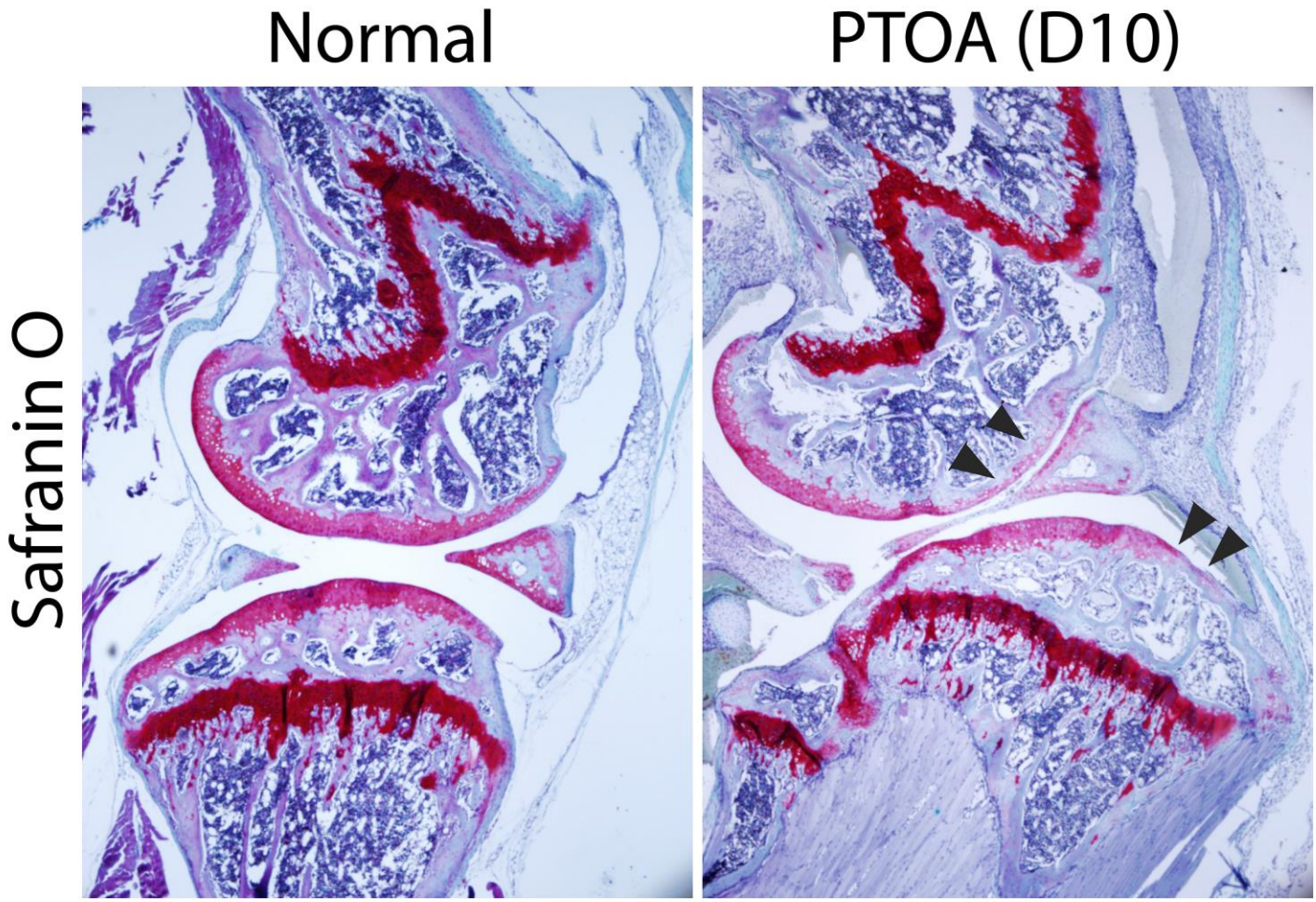

Figure 6: Comparison of the injured (right panel) versus contra-lateral control articular cartilages 10 days post surgery by Safranin-orange-stained staining. The damaged cartilage (Arrowhead) shows the thinner layer of the cartilage with less intensity.

One of the therapeutic approaches for tissue regeneration is to integrate the external intervention with endogenous capability, which provides a safer and longer term efficacy. It has been reported [8] that acute joint damage initiates a sequence of events not only can lead to progressive articular surface damage, and but also stimulate local cellular remodeling. To better understand the extent and capability of injured knee undergoing the remodeling, we analyzed some molecular markers such as Ki67, a marker for cell proliferation, after the articular fracture. As shown in **Fig. 7**, the surface of the damaged articular cartilage showed evidence of an increase in cell proliferation of chondrocytes, consistent with the literature that anabolic and catabolic response co-existed in the early phases of PTOA. Because the articular cartilage is a poorly vascularized tissue, separated from the bone, immediate healing from the articular fracture is impossible by the fact that mesenchymal stem cells from various sources can't be effectively delivered to the injured cartilage layers. In this regard, our data that the proliferative chondrocytes increase on the damaged surface of the cartilages in the acute phase of the PTOA may provide additional molecular targeting for the prevention and treatment of PTOA.

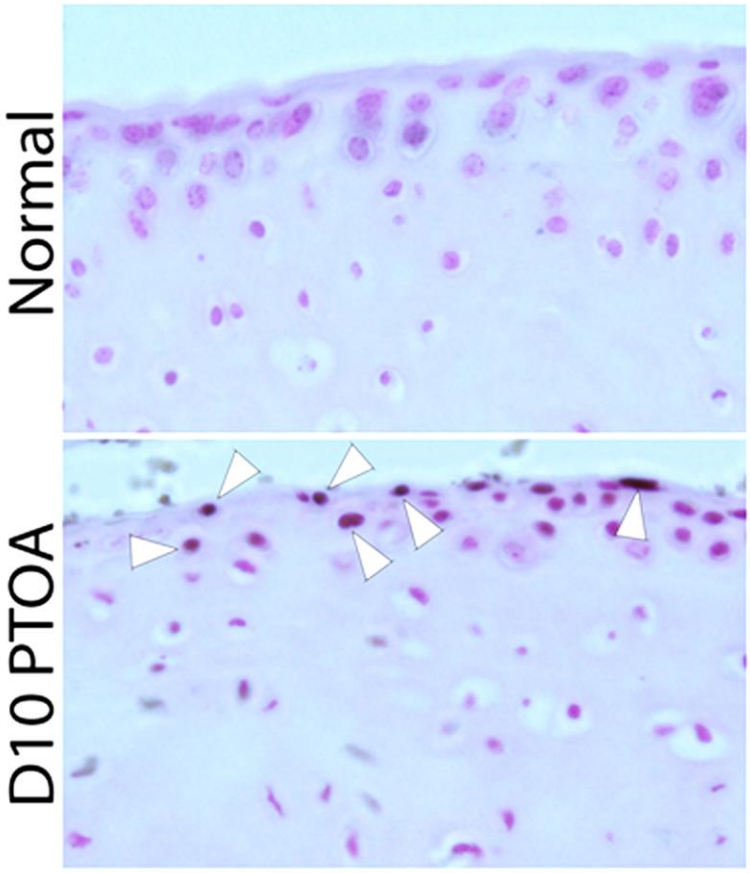

Figure 7: An increase in the number of proliferative chondrocytes by Ki67 expression (stained brownish, arrowheads) on the surface of damaged (bottom panel) versus contra-lateral (top) cartilages 10 days post fracture.

c. Progress towards Milestone 1, Specific Task 2: To demonstrate that the injected donor GFP-expressing M2 macrophages are recruited to and retained in the inflamed articular cartilage surface in the synovium of the PTOA knee joint for substantial period of time after intra-articular injection.

Current therapies are limited to pain management and inflammation reduction. However, they are not effective in halting the progression of the disease. Surgical options have been tried such as marrow stimulation (also known as “microfracture”), but the results are unsatisfactory and unable to restore a normal cartilaginous surface while the cost is very high. Recently, non-surgical options have been proposed and investigated in various preclinical and clinical settings. Various chondrogenic growth factor-based strategies are being investigated for promoting regeneration of articular cartilage [9]. The technical question and challenge is how to deliver these promising strategies to treat the PTOA knee.

Macrophage is a good candidate considering their active role in the lesion site. They will be recruited to the inflamed joints primarily when the joint is inflamed, and will dissipate when the synovial inflammation subsides. In addition, clinically, it is also relatively easy to isolate sufficient numbers of macrophages from human blood. Recent studies showed that macrophages can be further segregated into different sub-populations such as M1 and M2, functioning in inflammation and regeneration respectively [10]. However, in the IBD mouse model, our group showed that M2 rather than M1 macrophages were easily transduced by virus and stayed on inflamed intestine once implanted. Accordingly, in our PTOA mouse model, we used M2 over M1 macrophages to deliver therapeutic genes. To isolate M2 macrophages from bone marrow, we used the magnetic-bead selection by a specific antibody against mouse Gr1(+), a molecular marker for M2 macrophages. The purity of M2 macrophages isolated by this procedure was determined by FACS assay. A more than 90% of isolated cells was GR1 positive (+). We, subsequently, cultured these isolated Gr1 (+) cells in RPMI-1640 containing 10%FBS medium at the presence of mCSF (50 ng/ml) alone or with lipopolysaccride (LPS, 20 ng/ml) as a negative control, or IL4 (20 ng/ml) for 3 days. The molecular identifies of expanded cells were later determined by FACS assays. Our data showed that co-treated with mcsf and IL-4 yield the high % of M2 Macrophages determined by a more specific antibody against CD206. In contrast, the treatment with mCSF alone or mCSF +LPS reduced the % of M2 macrophages by >30% (**Fig. 8**).

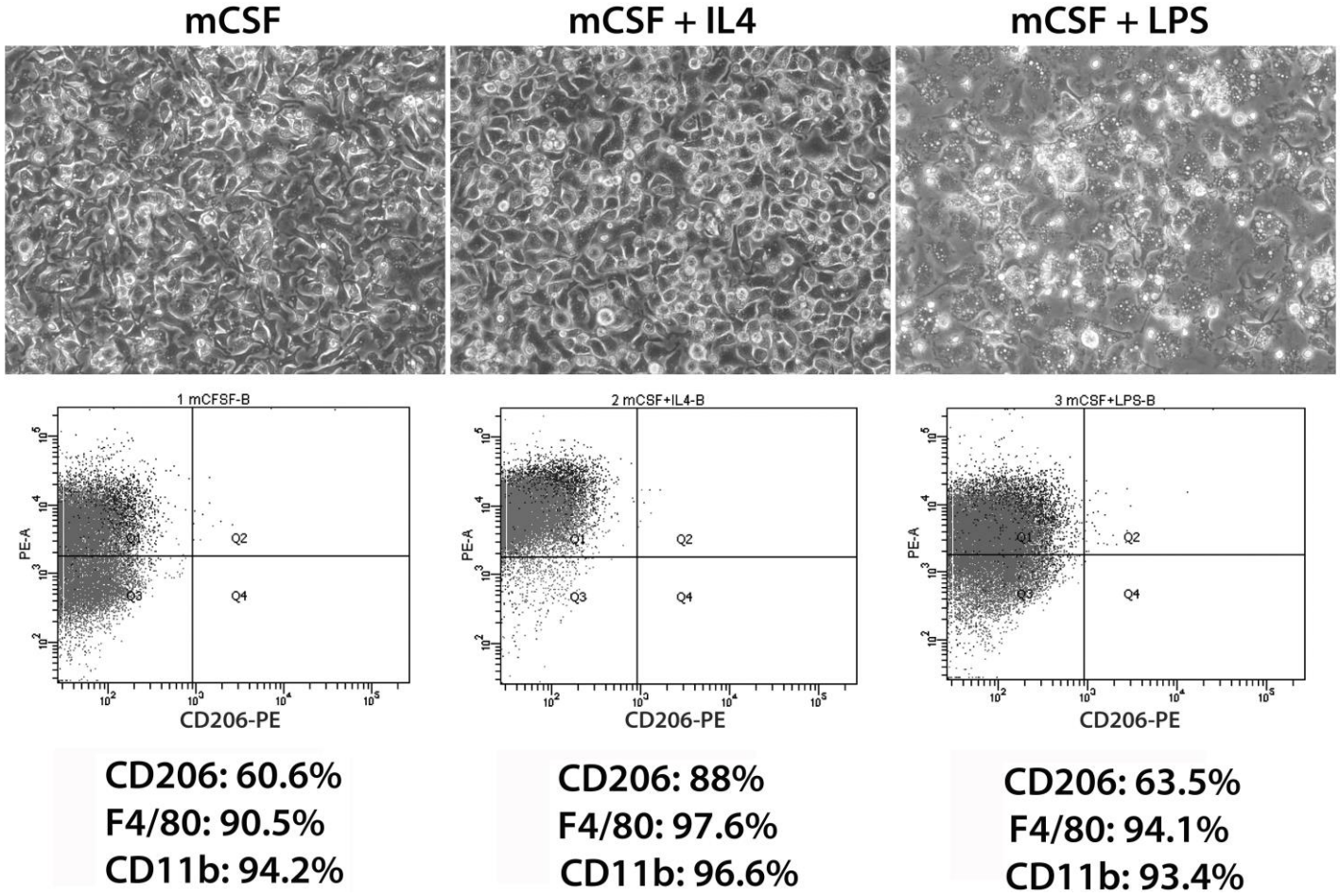

Figure 8: The expansion of M2 Macrophages *in vitro*. Quantitative FACS assay by different biomarkers specific for M2 macrophages including CD206, F4/80, CD11b, and Gr1.

Our goal is to apply the macrophage-based adoptive gene transfer strategy to prevent the progression of PTOA. Up to our knowledge, it's the 1st time to apply the ectopic macrophages to the PTOA joint. Therefore, there are a lot unknown about the fate of these injected macrophages in vivo bathed in the synovial fluid of injured knee. We need to examine how long the grafted macrophages will survive in the injured environment, and whether they will integrate into the lesion site and maintain their identities, and decide how often the injection should be performed to pursue the best effect.

To accomplish the goals, we used Faxitron to validate the injection site inside the joint as shown in **Fig.9** (top). After injection of an appropriate volume of green histological dye, the delivery was successful by the fact that green dye after delivery was restricted in the knee joint (**Fig.9**, bottom).

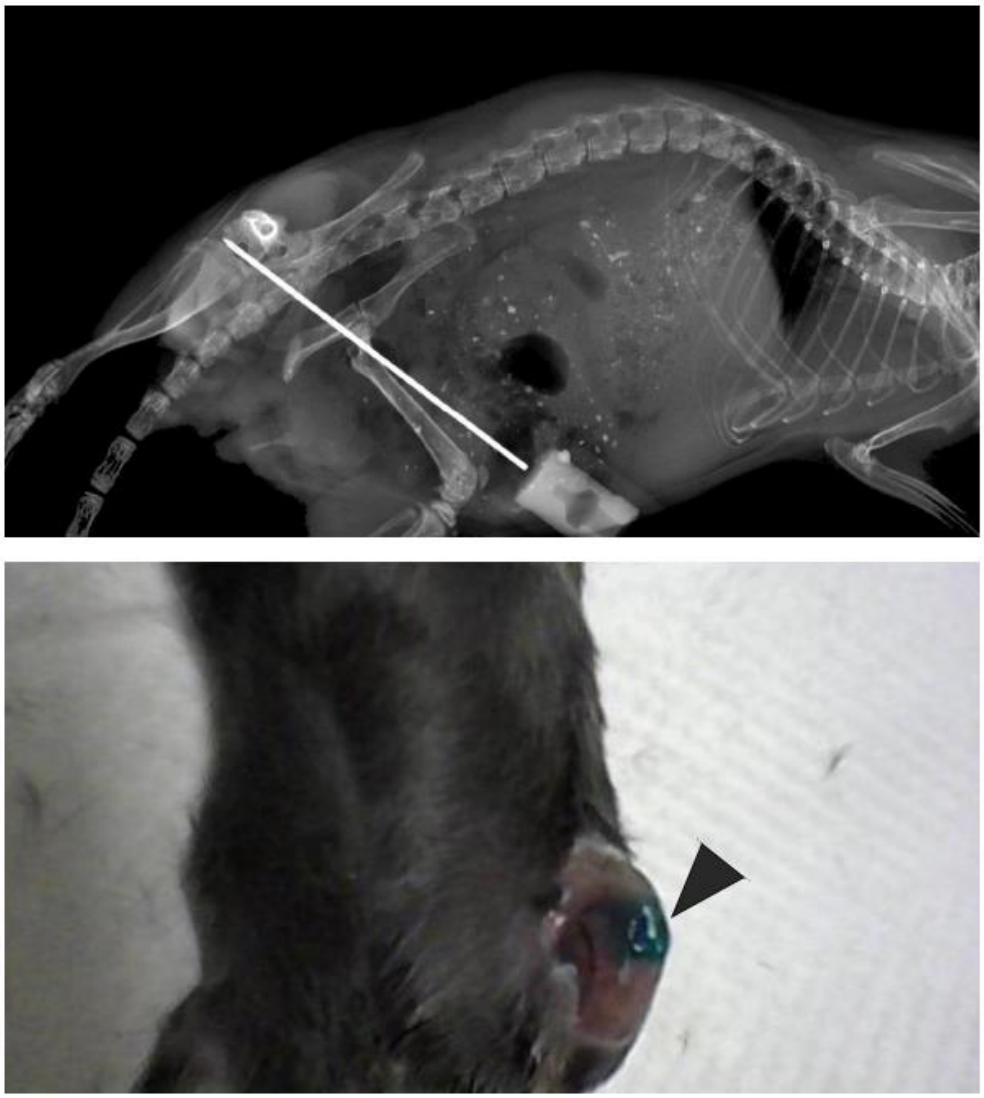

Figure 9: Faxitron X-ray machine guided knee injection was applied in pilot studies to validate the injection site inside the joint (upper panel). Green histological dye can be visualized surrounding the knee after one shot of injection in lower panel (arrowhead).

After the injection protocol was established, we next sought to determine the fate of injected macrophages implanted in the PTOA knee joint. We isolated GFP-expressing macrophages using the established GR1 antibody/magnetic bead isolation protocol from global GFP transgenic mice (ubi-GFP Tg). After expanded in cultures for three days at the presence of mCSF (50 ng/ml), these GFP-expressing macrophages (~1 x10^6 per joint) were implanted into the PTOA knee joints at day 4 post fracture. The knee joints (n=2 per time point) were collected at 3, 10, and 17 days post implantation and immediately fixed and frozen at -80C. Series of cryostat thin sections were obtained and mounted onto slides for the evaluation under a fluorescence microscope. We found that GFP (+) donor cells were sustained in the knee joints and surrounding connective tissue and muscle layer in the early time point (e.g., 3 days post injection) (**Fig.10**, top). However, 10 days post implantation, very few GFP (+) cells were detected around the PTOA joint (**Fig.10**, bottom), suggesting that the majority of implanted macrophages were gone 10 days after implantation. To examine the identity of those survived donor cells, we developed a double fluorescent staining protocol on frozen thin sections from a separate experiment. A large portion of GFP (+)GR1(+) macrophages were sustained 3 days after implantation (Fig.11), suggesting that a substantial number of the M2 macrophages were survival in the inflamed fluid in the early phase of PTOA. On the basis of the preliminary studies, we have successfully established the implantation protocol for the PTOA joints that is one injection per week and per million of macrophages per injection.

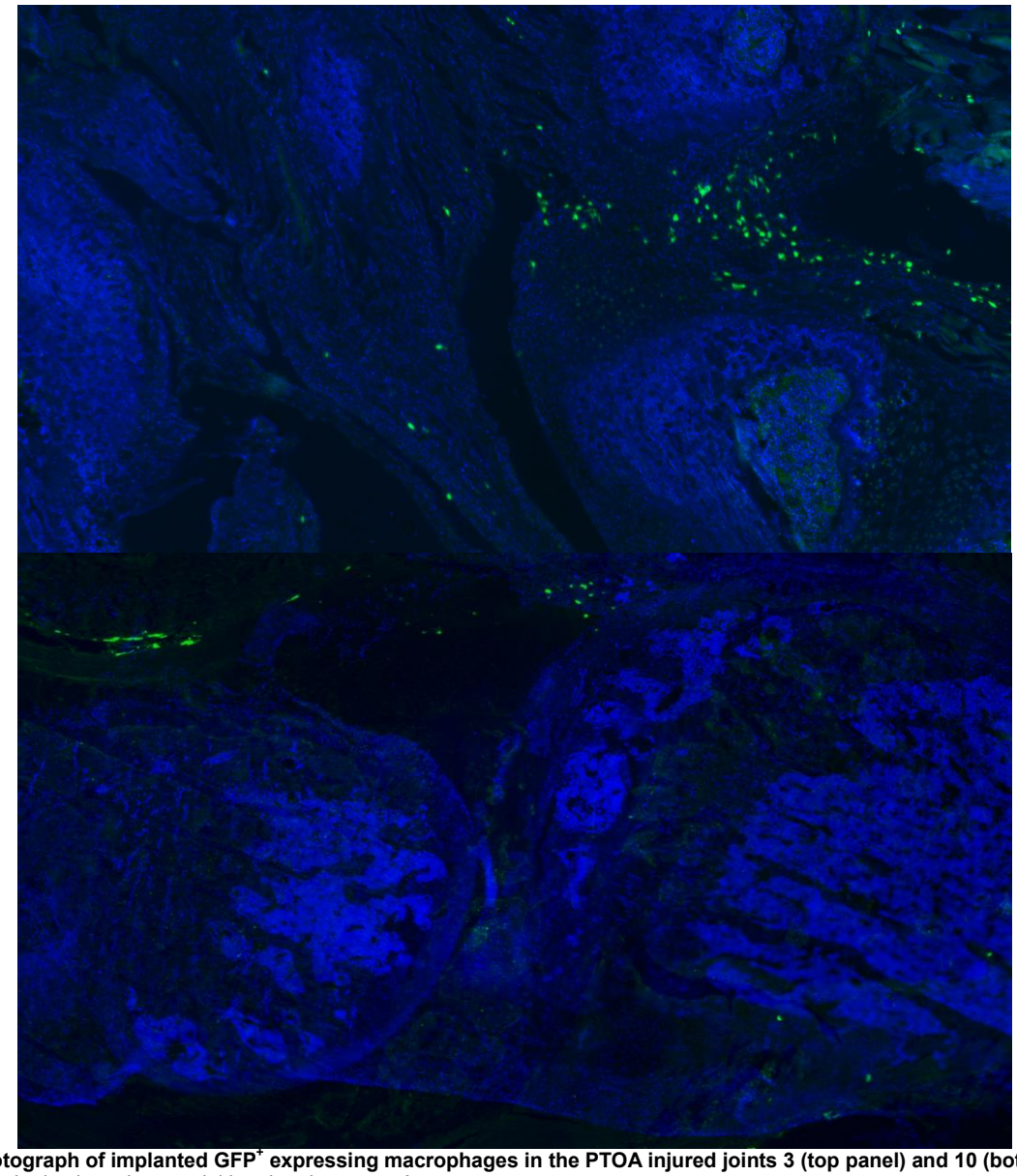

Figure 10: The photograph of implanted GFP^{+} expressing macrophages in the PTOA injured joints 3 (top panel) and 10 (bottom panel) days post implantation. The implantation was initiated at day 4 post fracture.

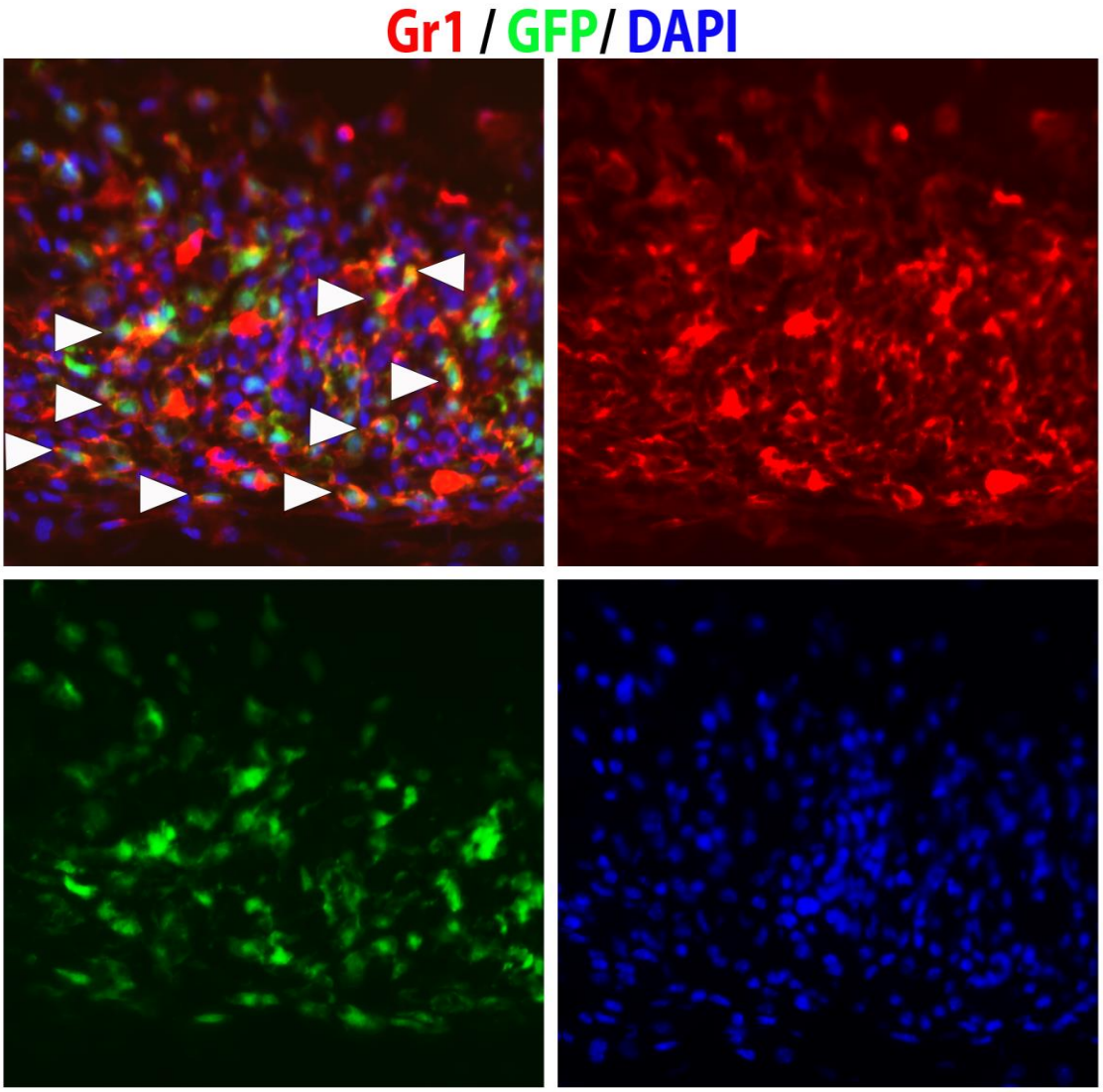

Figure 11. The photograph of the implanted macrophages expressing GR1 and/or GFP. Gr1 was visualized by red fluorescent whereas implanted macrophages are visualized by green fluorescent. The co-expression was indicated by yellow fluorescent.

d. **Progress toward Milestone 2, Specific Task 1: To construct the required lenti-viral based vectors that can effectively express murine IL-1ra, TGFβ3, or GFP marker genes.**

We have recently initiated the molecular cloning of the murine IL-1ra and TGFβ3 genes by long PCR approach using isolated mRNA from macrophages of C57BL/6J mice. The PCR products are currently being sequenced. Once the sequence is confirmed, we will begin generation of lentiviral-based vectors to express these two mouse genes in addition to the eGFP marker gene. The viral vector will then be used to transduced M2 macrophages to ensure that they can produce the gene products of interest as described in Milestone 2, Specific Task 2.

IV. Key Research Accomplishments:

- We have completed the required regulatory review and approval for use of animal subjects in this research from our IACUC and DoD animal Care and Use Review Office (ACRUO). We have also recruited a half-time research staff to assist us to perform the proposed research.
- We have successfully developed a closed intra-articular tibial plateau fracture model of PTOA in C57BL/6J mice.
- We have demonstrated that the injured knee joint but not the intact contralateral knee joint of the test animals yielded clear micro-CT and histological evidence for development of PTOA in as early as 4 weeks.

- We have also developed methods for isolation, expansion, implantation into the synovium of knee joint of M2 macrophages.
- We have also demonstrated that the implanted M2 macrophages are recruited to the inflamed areas of the injured knee joints.
- We are in the process to generate lenti-viral vectors expressing IL-1ra or TGFβ3 gene for use in the implantation experiment in next year of this project

V. Conclusion:

We have successfully accomplished all Specific Tasks of Milestone 1. We are making good progress towards both Specific Tasks of Milestone 2. We are now in position to carrying the proposed work in Milestones 3 and 4, which is to assess the clinical efficacy of the autologous macrophage-based anti-catabolic and pro-chondrogenic combination adoptive gene therapy for treatment and/or prevention of PTOA.

VI. Publications, abstracts, and presentations:

There is none to report at this time.

VII. Inventions, patents, and licenses:

None.

VIII. Reportable outcomes:

None

IX. Other achievements:

None

X. References

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XII. Appendices:

None.