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

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

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 (TGF-β3) 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 c) chondrogenic gene (TGF-β3) in the inflamed areas within the synovium of the PTOA joint; and 2) the IL-1ra or IL-1β shRNA and TGF-β3 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 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 syngeneic mouse macrophages into the PTOA synovium will result in confined and sustained production of large amounts of the transgenes (i.e., IL-1ra and TGF-β3) 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/TGF-β3 combination adoptive gene therapy on the suppression the inflammation-induced degradation of articular cartilage (and/or erosion of subchondral bone) and on the stimulation of the TGF-β3-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). Our data indicated that a macrophage-based adoptive gene transfer strategy provided a confined, sustained, and effective therapeutic tool to treat PTOA. In conclusion, our work demonstrates a novel approach for the prevention and treatment of PTOA.

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1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

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 costs of OA annually in the U.S. alone is close to $100 billion, of which nearly 50% is from lost earnings [2, 3]. Military personnel has 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 training and 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 currently no cure for PTOA or OA. Treatment options vary, including physical therapy, lifestyle changes, orthopedic bracing, and medications. Medications can help reduce inflammation in the joint to reduce pain, and may slow the progress of joint damage [5]. Joint replacement surgery may also be required in eroding forms of OA. This project seeks to obtain proof-of-concept type of evidence for the feasibility for and assess efficacy of an innovative autologous M2 macrophage-based anti-catabolic and pro-chondrogenic combination adoptive gene therapy for treatment of PTOA. This project has two specific hypotheses: 1) the autologous macrophage-based adoptive gene transfer strategy can effectively deliver and confine expression of an anti-catabolic gene, such as IL-1ra (or IL-1β shRNA) along with a chondrogenic gene, such as TGFβ3, in 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.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Osteoarthritis; Post-traumatic; IL1ra; TGF-β3; Transduction, Adoptive therapy, Macrophages, Chondrocytes; Articular Cartilage; Regeneration; Mice

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.

**What were the major goals of the project?**

*List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.*

The primary goal of this project was to obtain proof-of-concept evidence for the prevention and treatment using this novel macrophage-based adoptive gene transfer approach. There were two Specific Objectives:

**Objective 1.** To demonstrate that macrophage-based adoptive gene transfer strategy can effectively deliver and yield confined, sustained expression of transgenes in the inflamed synovia of the PTOA.

**Objective 2.** To demonstrate that the M2 macrophage-based IL-1ra (or IL-1β shRNA) and TGF-β3 combination adoptive gene therapy can effectively treat and prevent PTOA in a mouse PTOA model.
What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

**Technical Objective 1-a. To establish a mouse PTOA model:**

To test the feasibility and efficacy of M2 macrophage-based cell therapy for PTOA, it is necessary to have a valid animal model of PTOA for testing. Accordingly, the first task for this project was to establish a mouse PTOA model.

PTOA is generally caused by sudden application of mechanical force (impact) to the articular surface, which may or may not cause significant damages. Greater local tissue damages, such as chondrocyte death and matrix disruption, are the common consequences of high-energy impact or injury. Accordingly, we decided to establish a previously report intra-articular plateau fracture mouse model of PTOA [6] in the knee of C57BL/6J (B6) mice of 8-10 weeks for use in this project. 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.

![Fig. 1: The intra-articular tibial plateau fracture model.](image)

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 fractures, we used an Instron mechanical 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 injured knee, as opposed to the contra-lateral knee, displayed evidence for intra-articular bleeding, a classical feature of clinical PTOA.

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

**Fig. 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.
Fig. 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 mixtures of immune cells, dead chondrocytes, proteinases/cytokines, and degraded cartilage (Fig. 4). To confirm development of PTOA in our mouse model, we scanned the injured knee 12 weeks post-surgery by µCT. As shown in Fig. 5, bone spurs (small growths called osteophytes) grown on the edges of the joint was found in the PTOA but not the intact contra-lateral joint.

Fig. 5. 3-D images of inflamed knees 12 weeks after surgery by µ-CT.

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

Technical Objective 1-b. Establishment of the histological methods for assessing structural, cellular and molecular changes after PTOA:

Recent study by Anderson et al [7] suggests that development of PTOA can be segregated into two major phases that include 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 is critical and necessary. Histology is the common tool to evaluate the cellular and structure changes during the development of PTOA.
To study the progression of the pathophysiology of PTOA, we evaluated the injured joints versus intact 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 proteoglycan-enriched cartilage). As shown in Fig. 6, degradation of articular cartilage (stained in red) was observed in the injured but not the intact contra-lateral 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.

Fig. 6: Comparison of the Safranin-orange-stained articular cartilage in the injured joint (right panel) versus the contra-lateral control joint at 10 days post-surgery. The damaged (degraded) articular 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 to stimulation of 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 showing that anabolic and catabolic response co-existed in the early phases of PTOA. Because the articular cartilage is a poorly vascularized tissue and is separated from the bone, an immediate healing of articular fracture is impossible by the fact that mesenchymal stem cells from various sources cannot be effectively migrated to the injured cartilage layers for regeneration. In this regard, our data that the proliferative chondrocytes increase at the damaged surface of the cartilages in the acute phase of the PTOA may provide an additional molecular target for prevention and treatment of PTOA.
Fig. 7: An increase in the number of proliferative chondrocytes by Ki67 expression (arrowheads) on the surface of damaged (bottom panel) versus contra-lateral (top) cartilages 10 days post-fracture.

Technical Objective 1-c. Isolation and expansion of M2 macrophages in vitro

There is no cure for PTOA (or OA). 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 biologic 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 specifically and directly to the site of the PTOA joints.

PTOA is a disorder with chronic inflammation. Macrophages are known to play an active anti-inflammatory role at the injury site. Thus, macrophages could be an excellent targeting vehicle to deliver biologic agents to inflamed site, such as site of PTOA. Accordingly, 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 relatively easy to isolate sufficient numbers of autologous macrophages from the patient’s own 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 lipopolysacceride (LPS, 20 ng/ml) as a negative control, or IL4 (20 ng/ml) for 3 days. The molecular identities of expanded cells were later confirmed by FACS. Our data showed that co-treated with mCSF and IL-4 yield 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:** The expansion of M2 Macrophages in vitro. Quantitative FACS assay by different biomarkers specific for M2 macrophages including CD206, F4/80, CD11b, and Gr1.

**Technical Objective 1-d. Establishment of the M2 macrophage implantation protocol.**

Our goal is to apply the M2 macrophage-based adoptive gene transfer strategy to prevent the progression of PTOA. To our knowledge, this is the first time that one applies ectopic macrophages to the PTOA joint as a therapy. Therefore, there are a lot of unknown about the fate of these injected macrophages in vivo bathed in the synovial fluid of injured knee. We needed to examine how long the administered macrophages would survive at the injured joint, and whether they would engraft into the lesion site and still maintain their identity. This information will help us to decide how often the injection should be applied in order to achieve an optimal biological effect.

To reach these goals, we used Faxitron to validate the injection site inside the joint (as shown in the top panel of Fig. 9). After injection of an appropriate volume of green histological dye, the delivery was shown to be successful by the fact that the green dye after delivery was restricted in the knee joint (Fig. 9, bottom).
Fig. 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 determined the fate of the injected macrophages inside the PTOA knee joint. We isolated GFP-expressing M2 macrophages from global GFP transgenic mice (ubi-GFP Tg) using the established GR1 antibody/magnetic bead isolation protocol. After expanded in cultures for three days in the presence of mCSF (50 ng/ml), these GFP-expressing M2 macrophages (~1 x 10^6 per joint) were injected 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-injection and immediately fixed and frozen at -80°C. 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 found constantly in the knee joint and surrounding connective tissue as well as muscle layers during the early time points (e.g., 3 days post injection) (Fig. 10, top). However, 10 days after implantation, very few GFP (+) cells were detected around the PTOA joint (Fig. 10, bottom), suggesting that implanted macrophages were sustained until 10 days after injection. 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 GR1+GFP+ macrophages were 3 days after injection (Fig. 11), suggesting that a substantial number of the M2 donor macrophages were survival in the inflamed fluid in the early phase of PTOA. On the basis of the preliminary findings, we conclude that we have successfully established the protocol of implanting M2 macrophages into PTOA joints (i.e., one injection every two weeks and one million of macrophages per injection).

**Fig. 10:** The photomicrograph 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.
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Fig. 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.

Technical Objective 1-e. Establishment of lenti-viral based transduction of M2 macrophages protocol.
To generate mouse IL-1ra and TGF-β3 cDNAs, we purchased a mouse cDNA clone for each gene from Origene (Rockville, MD). We designed a pair of forward and reverse PCR primers that contain poly-A tails, AsisI or PMEI restriction enzyme site (forward: GCGATCGC; reverse: CAAATTTG), kozak sequence (GCCACCACC) and anti-sense sequence (15 to 30 bases). A unique DNA sequence of 527 bp (IL-1ra insert) and 1286 bp (TGF-β3 insert) was generated by PCR. To sub-clone these two inserts into a pRRLsin-cPPT-SFFV-X-hPGK-GFPpre plasmid (e.g., pSFFV-X), we first digested pRRLsin-cPPT-SFFV-cox2 -hPGK-GFPpre plasmid (pSFFV-cox2) with restriction enzymes, AsisI and PMEI, to remove cox2. As shown in Fig. 12, cox-2 (~2 kb) and pSFFV-X (~7.8 kb) are
separated by the restriction enzyme digestion. Sticky ends on these two IL-1ra and TGF-β3 inserts were made by AsisI/PMEI restriction enzyme digestion. To clone the inserts into pSFFV-X plasmid, we incubated insert and backbone in the presence of T4 ligase (Promega) at 16°C overnight. XL2-Blue Ultracompetent cells (Agilent Technologies) were infected with the ligation mix in the presence of antibiotics. Several individual colonies were randomly picked up and expanded in a LB medium at the presence of antibiotics. DNA was purified afterwards and positive clones were identified by restriction mapping. Specifically, we digested purified DNA with AsisI and PMEI and loaded digestion mix into an agarous gel. As shown in Fig. 13, positive clones were identified by the presence of a unique size (527 bp for IL-1ra and 1286 bp for TGF-β3) of DNA fragments. To further verify the two positive clones, we digest them as well as pSFFV-cox2 plasmid with KpnI. As shown in Fig.14, the predicted size of DNA products (2427 and 3186 bp) are present, confirming the insertion of IL-1ra and TGF-β3.

Fig. 12. The image of the inserts of IL1-ra (lane 2), TGF-β3 (lane 3), and cox-2 (lanes 4 and 5) as well as the backbone of pSFFV-cox2 (lanes 4 and 5) after AsisI/pMEI restriction enzyme digestion. The gel bands containing IL-1ra (~527 bp), TGF-β3 (1286 bp), and pSFFV-X (7400 bp) are collected for subsequent ligation experiment. pSFFV-cox2 in a format of circular (without restriction enzyme digestion, lane 1) or of linear (one restriction enzyme; lane1) are used to determine the outcome of single restriction enzyme digestion and confirm the accuracy of electrophoresis.

Fig. 13. The image of positive clones for IL-1RA (red, lane 1, 527 bp) and TGF-β3 (blue, lane 7, ~1286 bp). Individual colony is randomly selected and expanded in LB medium at 37°C for overnight. DNA was purified from each of expansion and digested with AsisI and pMEI. Positive clones are determined by the presence of 527 bp and 1286 bp for IL1-ra and TGF-β3, respectively.
Finally, pSFFV-IL-1ra and pSFFV-TGF-β3 were sent to McLab (South San Francisco, CA) for DNA sequencing. The sequencing results confirmed that two plasmids (e.g., pSFFV-IL1-RA and pSFFV-TGF-β3) contain the correct DNA sequence of the full length of IL-1ra and TGF-β3 cDNAs.

In summary, we successfully developed this IL-1RA and TGFβ3 containing plasmids (e.g., pSFFV-IL-1ra and pSFFV-TGF-β3) which were used for subsequent macrophage transduction.

**Milestone 2 Specific Task 2:** To confirm that the isolated mouse M2 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 (Technical Objective 1-b)

**Technical Objective 2-a: Establishment of lenti-viral based macrophage transduction protocol**

To generate TGF-β3 or IL-1RA containing lenti virus, we infected XL2-Blue competent cells (Agilent Technologies) with constructed pSFFV-TGF-β3 or IL-1RA plasmids to produce large amounts of plasmids, which were then purified with Endo-free plasmid isolation kit (Qiagen). After purification and verification by restriction enzyme mapping, pSFFV-TGF-β3 or pSFFV-IL-1RA plasmids were used to infect KEK 293 T cells along with VSV-G and Pax2 capsid plasmids using the calcium phosphate method [11]. The conditioned medium containing viral particles were collected during the first 60 hours, concentrated, aliquoted, and frozen at -80°C.

To test whether M2 macrophages can be effectively transduced with the lentiviral particles containing IL-1ra or -TGF-β3 we made, we first isolated bone marrow cells from adult C57BL/6J mice and cultured them for 3 days to eliminate attached cells. The unattached bone marrow cells were subsequently treated with mCSF for 3 days. These attached macrophages were then converted to M2 macrophages by IL-4, pSFFV-TGF-β3, IL-1ra, or pSFFV-GFP control (MOI=2 for each) viral particles was each added into the cultures of M2 macrophages (5x10⁵ cells/well) in the presence of protamine sulfate (8 µg/ml) for 12 hours. Cultures were then replaced with fresh conditioned medium containing MCSF/IL-4 for additional 3 days. The production of TGF-β3 or IL-1RA in the conditioned medium of the transduced macrophages was analyzed by commercial ELISA kits (MyBiosource). M2 macrophages transduced with viral particles containing pSFFV-TGF-β3 (Fig. 15) or IL-1RA (Fig. 16) exerted more than one- or three-fold increase in TGF-β3 or IL-1RA, respectively.
On the basis of our data, we conclude that macrophages after lenti virus based transduction produced and released sufficient amounts of TGF-β3 and IL-1RA protein.

We subsequently used an established protocol as described in above to implant IL-1RA or TGF-β3 expressing M2 macrophages. One million of transduced macrophages via lenti-viral transduction were implanted into the inflamed knees. Two weeks after implantation, GFP positive cells were visualized on top of inflamed knee by anti-GFP antibody (Rockland, Limerick, PA) immunostaining (Fig. 17), suggesting 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.
To demonstrate that the macrophage-based IL-1ra (or IL-1β shRNA) and TGF-β3 combination adoptive gene therapy can effectively treat and prevent PTOA in a mouse PTOA model, we implanted 0.6 to 1.0 million of infected macrophages per injection to the inflamed knees on day 7 and day 18 post surgery. The knees were evaluated 2.5 weeks after the last injection. As shown in Fig. 18, there was a loss of articular cartilage of the knees with the implantation of transduced macrophages expressing GFP control or IL-1RA. However, there was substantial improvement in the amount of articular cartilage when the inflamed knee was implanted with macrophages expressing both TGF-β3 and IL-1RA. A lack of the effect of IL-1RA in the prevention of articular cartilage degradation is unexpected that requires some work to investigate whether it is due to the secondary effect of IL-1RA mediated IL-1 blockage on the activity, migration, or survival of implanted macrophages [12]. Additional work will be necessary to determine these possibilities. Nevertheless, the effect of TGF-β3 delivered by implanted macrophages on the cartilage synthesis in the early progression of PTOA seems promising. The functional role of TGF-β3 in the late stage of PTOA progression was shown in Fig. 19. The inflamed knees were implanted twice with either GFP control or TGFβ3 expressing macrophages 8 weeks after surgery. The knees were then examined one week after the last injection. The inflamed knee implanted with TGF-β3 expressing macrophages showed a substantial increase in the amount of cartilage in the inflamed knee in comparison with the knee implanted with GFP-expressing macrophages. Although the amount of articular cartilage is not as great as the contralateral control knee the outcome of the locally implanted macrophages which delivered TGF-β3 to the inflamed cartilage showed promising.

In conclusion, our work demonstrates a novel approach for the prevention and treatment of PTOA. The outcome of the proposed adoptive approach requires substantial work to confirm by increasing number of animals and by recommended scientific method [13].
Fig. 18. The effect of locally injection of GFP-, IL-1RA, and/or TGF-β3 expressing macrophages in the prevention of PTOA. Safranin O stained paraffin sections of fractured versus contralateral control knees were compared. The cartilage is stained orange in color. Infected macrophages were implanted on day 6 post surgery.
Fig. 19. The effect of locally injection of GFP- or TGF-β3-expressing macrophages in the treatment of PTOA. Infected macrophages were implanted on day 61 post surgery.

REFERENCES

3. Arthritis: The Nation’s Most Common Cause of Disability Centers for disease prevention and health promotion. At a Glance 2013, Centers for Disease Control and Prevention
What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. “Training” activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. “Professional development” activities result in increased knowledge or skill in one’s area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to Report.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state “Nothing to Report.”
Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Nothing to Report.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

**What was the impact on the development of the principal discipline(s) of the project?**
*If there is nothing significant to report during this reporting period, state “Nothing to Report.”*

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

PTOA/OA is a disorder with chronic inflammation. The premise of our project is to take advantage of the well-known homing property of M2 macrophages to sites of inflammation to use M2 macrophage as the cell vehicle to target deliver therapeutic biologics to the inflamed sites of PTOA for treatment or prevention of PTOA or OA. The immediate objective of this project was to gather test-of-principle evidence for the feasibility of the approach to deliver biologics to the PTOA site. The secondary objective was to test whether target delivery of an anti-catabolic agent, i.e., IL-1ra, in addition to a pro-anabolic growth factor, i.e., TGF-β3, with the M2-macrophage approach would prevent degradation of articular cartilage during development of PTOA or regenerate articular cartilage after PTOA is established. Our work thus far is very encouraging and strongly support the feasibility of the M2-macrophage based therapy. While the combination therapy of IL-1ra and TGF-β3 appears to have some protective and regenerative protential, the therapy might have better effects if more appropriate anti-catabolic and/or anabolic factors are used. Accordingly, our future goals will search for more effective agents in addition to optimize this therapy. If our effort is successful, this therapy could be developed into an effective therapy for PTOA/OA, which currently has no cure.

**What was the impact on other disciplines?**
*If there is nothing significant to report during this reporting period, state “Nothing to Report.”*

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Although our goal for this M2-macrophage adoptive therapy was intended for treatment or prevention of PTOA/OA, this therapy can easily be adopted to other inflammation-related disorders, including rheumatoid arthritis and other inflammatory-related bone diseases.

**What was the impact on technology transfer?**
If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:
- transfer of results to entities in government or industry;
- instances where the research has led to the initiation of a start-up company; or
- adoption of new practices.

Our research is still at the developmental phase. Our current effort focuses on tests of feasibility and efficacies. Once these objectives are accomplished, we will test address the safety issues. Accordingly, it is premature to consider technology transfer at this stage of our research. Therefore, there is nothing to report at this time.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:
- improving public knowledge, attitudes, skills, and abilities;
- changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or
- improving social, economic, civic, or environmental conditions.

Nothing to Report.

5. CHANGES/PROBLEMS: The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, “Nothing to Report,” if applicable:

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

During this report period, there is no changes in objectives and scopes, with the exception for our request of a 12-month no-cost-extension (which was approved by the DOD) to accomplish the remaining Technical Objectives.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

The funding and the project have ended. There will no further action or plans to be taken.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

During the funding period, we have encountered a significant delay in recruiting a part time post-doc fellow at the beginning of the funding period and an early departure of a technician during the no-cost-extension. These problems have somewhat impeded our overall progress of this project.

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

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

**Significant changes in use or care of human subjects**

Not applicable.

**Significant changes in use or care of vertebrate animals**

None.

**Significant changes in use of biohazards and/or select agents**

**PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state “Nothing to Report.”

None.

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  **Journal publications.** List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).

  Nothing to Report.
Books or other non-periodical, one-time publications. Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).

Nothng to Report.

Other publications, conference papers and presentations. Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.

Nothing to Report at this time. However, we plan to publish our results in the future.

- Website(s) or other Internet site(s)
List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to Report.

- Technologies or techniques
Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to Report.

- Inventions, patent applications, and/or licenses
Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.
• **Other Products**

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and/or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- data or databases;
- physical collections;
- audio or video products;
- software;
- models;
- educational aids or curricula;
- instruments or equipment;
- research material (e.g., Germplasm; cell lines, DNA probes, animal models);
- clinical interventions;
- new business creation; and
- other.

Nothing to Report.

6. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate “no change”.

**Example:**

<table>
<thead>
<tr>
<th>Name</th>
<th>Mary Smith</th>
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<tbody>
<tr>
<td>Project Role:</td>
<td>Graduate Student</td>
</tr>
<tr>
<td>Researcher Identifier (e.g. ORCID ID): 1234567</td>
<td></td>
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<tr>
<td>Nearest person month worked:</td>
<td>5</td>
</tr>
<tr>
<td>Contribution to Project:</td>
<td>Ms. Smith has performed work in the area of combined error-control and constrained coding.</td>
</tr>
<tr>
<td>Name:</td>
<td>Matilda Sheng</td>
</tr>
<tr>
<td>-----------------------</td>
<td>---------------------------------------------------</td>
</tr>
<tr>
<td>Project Role:</td>
<td>Principle Investigator</td>
</tr>
<tr>
<td>Contribution to Project:</td>
<td>Dr. Sheng led this project, planed experiments, supervised post-doc fellows, performed trouble shooting/data analysis, and wrote progress reports.</td>
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<tr>
<th>Name:</th>
<th>Kin-Hing W Lau</th>
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<tr>
<td>Project Role:</td>
<td>Collaborator</td>
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<tr>
<td>Contribution to Project:</td>
<td>Dr. Lau gave suggestion to technical problems and provided concept and interpretation</td>
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<th>Name:</th>
<th>David J Baylink</th>
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<tr>
<td>Project Role:</td>
<td>Collaborator</td>
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<tr>
<td>Contribution to Project:</td>
<td>Dr. Baylink gave critical comments on study design and data interpretation</td>
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<th>Name:</th>
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<td>Project Role:</td>
<td>Post-doc fellow (4 months)</td>
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<tr>
<td>Contribution to Project:</td>
<td>Dr. Xu (half-time) assisted PI to set up the protocols for fracture, isolation of macrophages, implantation, immunostaining, and wrote the first report.</td>
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<th>Name:</th>
<th>Biswanath Patra</th>
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<tr>
<td>Project Role:</td>
<td>Post-doc fellow (3 months)</td>
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<tr>
<td>Contribution to Project:</td>
<td>Dr. Patra assisted PI to perform fracture and evaluation of samples</td>
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<tr>
<th>Name:</th>
<th>Denise Rodriguez</th>
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<tbody>
<tr>
<td>Project Role:</td>
<td>Research Technician (3 months)</td>
</tr>
<tr>
<td>Contribution to Project:</td>
<td>Ms. Rodriguez embedded specimens and performed sectioning and histochemical staining</td>
</tr>
</tbody>
</table>
Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state “Nothing to Report.”

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to Report.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner’s contribution to the project (identify one or more)
• Financial support;
• In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);
• Facilities (e.g., project staff use the partner’s facilities for project activities);
• Collaboration (e.g., partner’s staff work with project staff on the project);
• Personnel exchanges (e.g., project staff and/or partner’s staff use each other’s facilities, work at each other’s site); and
• Other.

Nothing to Report.
7. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to https://ers.amedd.army.mil for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

8. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

None.