

SUSTAINED INTRA-ARTICULAR DELIVERY OF IL-1RA FROM A THERMALLY-RESPONSIVE ELASTIN-LIKE POLYPEPTIDE AS A THERAPY FOR POST-TRAUMATIC ARTHRITIS

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Abstract

Post-traumatic arthritis (PTA) is a rapidly progressive form of arthritis that develops due to joint injury, including articular fracture. Current treatments are limited to surgical restoration and stabilization of the joint; however, evidence suggests that PTA progression is mediated by the upregulation of pro-inflammatory cytokines, such as interleukin-1 (IL-1) or tumor necrosis factor- α (TNF- α). Although these cytokines provide potential therapeutic targets for PTA, intra-articular injections of anti-cytokine therapies have proven difficult due to rapid clearance from the joint space. In this study, we examined the ability of a cross-linked elastin-like polypeptide (xELP) drug depot to provide sustained intra-articular delivery of IL-1 and TNF- α inhibitors as a beneficial therapy. Mice sustained a closed intra-articular tibial plateau fracture; treatment groups received a single intra-articular injection of drug encapsulated in xELP. Arthritic changes were assessed 4 and 8 weeks after fracture. Inhibition of IL-1 significantly reduced the severity of cartilage degeneration and synovitis. Inhibition of TNF- α alone or with IL-1 led to deleterious effects in bone morphology, articular cartilage degeneration, and synovitis. These findings suggest that IL-1 plays a critical role in the pathogenesis of PTA following articular fracture, and sustained intra-articular cytokine inhibition may provide a therapeutic approach for reducing or preventing joint degeneration following trauma.

Keywords: Arthritis, murine model, cartilage, drug delivery, inflammation.

Introduction

Post-traumatic arthritis (PTA) is a progressive and degenerative form of arthritis that develops following joint trauma (Brown *et al.*, 2006). Most commonly, joint trauma can be incurred by excessive loading of a synovial joint, tears of the meniscus, ligament, or capsule, joint dislocation, or an articular fracture. Currently, 12 % of the 27 million Americans that suffer from osteoarthritis (OA) are believed to have PTA (Brown *et al.*, 2006; Lawrence *et al.*, 2008). An excess burden of PTA is observed in military populations, wherein OA after joint injury is the leading cause of disability that prevents soldiers from remaining on active duty (Patzkowski *et al.*, 2012). The rate of developing PTA in weight-bearing joints after combat injuries is 60 %, with a 100 % incidence rate for knee joints following trauma or injury (Rivera *et al.*, 2012). The total economic burden of PTA has been estimated to exceed \$3 billion in direct health care expenses annually (Brown *et al.*, 2006).

Of all types of joint trauma, intra-articular fractures most commonly lead to PTA, despite surgical restoration and stabilization of the joint (Buckwalter and Brown, 2004). Articular fractures are complex injuries that occur as a result of excessive, traumatic loading that can cause damage in the bone, soft tissue, synovium, and vascular regions (Cole *et al.*, 2009; Furman *et al.*, 2006). Following injury, inflammatory cytokines and chemokines are elevated in synovial fluid (Bigoni *et al.*, 2013; Cameron *et al.*, 1997; Elsaid *et al.*, 2009; Higuchi *et al.*, 2006; Irie *et al.*, 2003; Lewis *et al.*, 2013; Marks and Donaldson, 2005; Mountziaris and Mikos, 2008; Sward *et al.*, 2012). Of these cytokines, interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF- α) are typically found at the site of injury after trauma and aid in recruiting other cytokines and chemokines, such as IL-6 and IL-8, necessary to the healing process (Abramson and Yazici, 2006; Fazzalari, 2011; Fernandes *et al.*, 2002). However, IL-1 and TNF- α also exhibit a chondro-destructive effect on cartilage by stimulating the release of other cytokines and enzymes, such as matrix metalloproteinases and aggrecanases, that break down articular cartilage (Fukui *et al.*, 2001; Marks and Donaldson, 2005; McNulty *et al.*, 2013). Rapid upregulation of IL-1 and TNF- α was shown in a previous murine articular fracture study, with IL-1 β upregulated 750-fold hours after fracture, IL-1 α upregulated 7-fold one

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day after fracture, and TNF- α upregulated 13-fold one day after fracture (Lewis *et al.*, 2013).

The role of IL-1 and TNF- α in inflammatory arthritis has been extensively studied; currently, there are several commercially available cytokine inhibitors for the treatment of rheumatic diseases. Interleukin-1 Receptor antagonist (IL-1Ra, anakinra, Kineret[®]) is FDA approved for the treatment of rheumatoid arthritis (RA). IL-1Ra works by competitively binding to the IL-1 receptor (IL-1R) and attenuating the signaling associated with IL-1/IL-1R binding. Similarly, soluble tumor necrosis factor receptor II (sTNFR_{II}, etanercept, Enbrel[®]) is FDA approved for use with RA patients. sTNFR_{II} is a soluble fragment of the TNF receptor that competitively binds TNF- α and TNF- β in the extracellular space, thus preventing their binding to the membrane-bound TNF receptor. The results of past clinical studies have shown some efficacy in RA patients when IL-1Ra was delivered systemically (Bresnihan *et al.*, 1998; Fleischmann, 2006; Fleischmann *et al.*, 2003b; Fleischmann *et al.*, 2006b; Nuki *et al.*, 2002) and when sTNFR_{II} was delivered locally (Bliddal *et al.*, 2006; Boesen *et al.*, 2008; Fiocco *et al.*, 2010; Roux *et al.*, 2011) or systemically (Bathon *et al.*, 2006; Fleischmann *et al.*, 2006a; Fleischmann *et al.*, 2003a; Johnsen *et al.*, 2006; Kavanaugh *et al.*, 2008; Krieckaert *et al.*, 2012; Moreland *et al.*, 1999).

While local or systemic delivery of IL-1Ra or sTNFR_{II} has been established to provide efficacy against RA, less is known of the potential for local cytokine antagonism to modify disease or symptoms in patients affected by OA, particularly in isolated joints. For example, an intra-articular injection of IL-1Ra into the knee joint improved symptoms in patients with established OA over the course of 4 d (Chevalier *et al.*, 2005; Chevalier *et al.*, 2009), although the improvements with joint injection diminished to control levels over time. The short duration of the efficacy of IL-1Ra in this study was attributed to its brief intra-articular half-life, which is of the order of 2-6 h (Chevalier *et al.*, 2009). Nonetheless, if administered after joint trauma, a single intra-articular injection of IL-1Ra was shown to improve short-term knee pain and function scores (Kraus *et al.*, 2012). For OA, the use of etanercept has not been documented; however, systemic administration of two other anti-TNF drugs (infliximab and adalimumab) has been shown to provide a modest improvement in symptoms for patients with hand OA (Guler-Yuksel *et al.*, 2010; Magnano *et al.*, 2007). Because of the potential pro-inflammatory role of IL-1 and TNF- α in arthritis, and the success of both IL-1 and TNF inhibitors, subsequent studies have included groups that administer both drugs at the same time. For example, in a TNF-induced murine arthritis model, the systemic administration of IL-1Ra, anti-TNF, or a combination of both IL-1Ra and anti-TNF was examined. Synovial inflammation, bone erosion, and cartilage degeneration were blocked by both anti-TNF and combination therapy (Zwerina *et al.*, 2004). However, such an approach has not been examined as a therapy for intra-articular fracture, which involves a complex cascade of bone and soft tissue damage and inflammation leading

to PTA (Furman *et al.*, 2006; Furman *et al.*, 2007; Lewis *et al.*, 2013). Our previous research indicated that a single intra-articular injection of IL-1Ra reduced, but did not completely eliminate the manifestations of PTA after a closed articular fracture (Furman *et al.*, 2014b; Mangiapani *et al.*, 2012).

Local intra-articular administration to the knee joint through use of sustained-release depots is advantageous for the ability to deliver relatively high total drug doses, while minimizing the peak drug concentration in systemic circulation and allowing therapeutic drug doses over prolonged times (Evans *et al.*, 2014; Larsen *et al.*, 2008). Previous work by Gorth and co-authors have shown that the release of IL-1Ra from poly(lactic-co-glycolic acid) microspheres helped to reduce degradation of the nucleus pulposus caused by IL-1 β (Gorth *et al.*, 2012). Elastin-like polypeptides (ELPs) have demonstrated value as injectable drug depots that can provide for local, sustained release of drugs in the knee joint (Allen *et al.*, 2011; Allen *et al.*, 2010; Shamji *et al.*, 2007). ELPs are pentapeptide sequences from native human elastin (V-P-G-X-G, where X is any amino acid) (Urry, 1992; Urry *et al.*, 1998) with a history of medical applications that illustrate their biocompatible and non-immunogenic properties (Adams *et al.*, 2009; Betre *et al.*, 2006a; Chilkoti *et al.*, 2002a; Chilkoti *et al.*, 2002b; MacEwan and Chilkoti, 2009; Megeed *et al.*, 2002; Nettles *et al.*, 2010; Tejada-Montes *et al.*, 2012). ELPs undergo an inverse phase transition, wherein they are soluble in aqueous solutions below a specific transition temperature (T_t) and become insoluble above T_t (Urry, 1997). This phase transition causes the formation of self-associating particles that can entrap drug when the temperature is raised above T_t ; when T_t is targeted below body temperature, the injected ELP can spontaneously form a depot for prolonged drug release in the body (Adams *et al.*, 2009; Betre *et al.*, 2006a; Meyer and Chilkoti, 2002; Shamji *et al.*, 2007). While prior studies have largely relied upon conjugation of drug to the ELP prior to depot formation, entrapment of drug in ELP is an alternate possibility. Adams and co-workers found ELP entrapment of antibiotics may be improved following inter-ELP crosslinking that promotes cohesion in the depot formation (Adams *et al.*, 2009). This strategy is attractive also for large molecule protein drugs for the benefit that entrapment preserves the structure and function of the native protein drug.

For this study, we hypothesized that sustained local (intra-articular) inhibition of IL-1, TNF- α , or both IL-1 and TNF- α together would prevent the development of PTA following articular fracture. The objective of this study was to locally deliver a drug-containing ELP depot to inhibit intra-articular inflammation following trauma using a closed articular fracture model in the tibial plateau of mice. The severity of arthritic changes in mouse knee joints was assessed from histologic analysis of joint tissues, bone morphologic changes from microCT, and biomarkers from serum and synovial fluid, and compared amongst single injections of depot-delivered IL-1Ra, sTNFR_{II}, or the dual combination to identify the most promising treatment option.

Materials and Methods

ELP production and encapsulation

One ELP sequence, VPGKG(VPGVG)₁₆-102, termed KV16-102 (MW 42.7 kDa), was chosen for this study as described previously (Adams *et al.*, 2009; Meyer and Chilkoti, 2002; Trabbic-Carlson *et al.*, 2003). In brief, an expression vector containing the coding ELP gene was transformed into *E. coli* BLR(DE3) (Novagen, Madison, WI, USA) for protein hyperexpression using previously established procedures. ELP was purified by successive thermal cycling, passed through a sterile filter, and resuspended in PBS at 150 mg/mL before use. For each ELP drug-loading construct to be used in animal studies, 80 μ L of ELP solution was mixed with an organophosphorous cross-linker, β -[tris(hydroxymethyl) phosphino]propionic acid (THPP), an amine-reactive crosslinker at a 1:1 molar ratio of ELP amines to THPP sites (Lim *et al.*, 2007; Lim *et al.*, 2008; Nettles *et al.*, 2010). The mixture was placed on a shaker at 4 °C for 30 min, and then allowed to crosslink at 37 °C for 24 h. The cross-linked ELP (xELP) was lyophilized, quenched in a sterile cell culture hood, and then re-lyophilized. At 37 °C, it has been shown that these cross-linked ELPs will undergo a transition to a gel-like phase with a change in opacity of the ELP construct, thus preserving much of the thermally-induced transition properties of the unmodified ELPs (Adams *et al.*, 2009). Drug loading into lyophilized xELP constructs was performed by rehydrating the xELP in 80 μ L of a drug-containing solution (or phosphate-buffered saline (PBS) for control) to yield the final drug concentrations in a 6 μ L injection as follows: xELP[IL-1Ra] = 0.45 mg of IL-1Ra; xELP[sTNFR2] = 0.15 mg of sTNFR2; xELP[IL-1Ra+sTNFR2] = 0.45 mg of IL-1Ra and 0.15 mg of sTNFR2; xELP[PBS] received only PBS. Constructs for both the *in vitro* and *in vivo* experiments were prepared the same, with the exception that the constructs prepared for the *in vivo* experiment were done under sterile conditions.

In vitro release of drug from xELP

In vitro drug release profiles of both IL-1Ra and sTNFR2 were quantified as described below. Lyophilized xELP depots (12 mg ELP protein) were rehydrated in either PBS (xELP[PBS]), IL-1Ra (xELP[IL-1Ra], anakinra), or sTNFR2 (xELP[sTNFR2], etanercept) and incubated in either PBS or 10 % serum-containing medium (10 % fetal bovine serum (FBS) in PBS) to determine the release of each drug over 7 d. For samples in PBS, protein absorbance at 280 nm was used to quantify the concentration of released drug. In order to account for any release of uncross-linked ELP, the absorbance of xELP[PBS] was measured at each time point to serve as an experimental "blank." The molar extinction coefficients for both IL-1Ra and sTNFR2 were used at each time point, along with the Beers-Lambert Law in order to quantify drug concentrations in the supernatant at each time point. For the serum-containing medium, absorbance could not be used to quantify drug release, so human IL-1Ra and sTNFR2 ELISAs were used (DRA00B and DRT200, R&D Systems,

Minneapolis, MN, USA). For each ELISA, xELP[PBS] samples were measured as controls to ensure that there was no background interference from ELP fragments released by esterases or proteases in FBS.

In vivo drug release assays

In vivo release profiles for both IL-1Ra and sTNFR2 were obtained using serial collections of serum from mice. Blood was collected at day 1, 5, 14, 21, 28, 42 and 56 following fracture. All serum samples from a single group and collection day were pooled ($n = 6-11$ per group per time point) in order to obtain sufficient fluid volumes for detection of IL-1Ra (minimum 100 μ L of sample) or sTNFR2 (minimum 20 μ L of sample) using commercially available human ELISA kits (DRA00B and DRT200, R&D Systems). For statistical purposes, a value of $\frac{1}{2}$ the lowest level of detection (LLOD) was used for any value that was below the level of detection (IL-1Ra: 3.1 pg/mL and sTNFR2: 0.3 pg/mL). The reported mean intra- and inter-assay coefficients of variation for IL-1Ra are 5.3 % and 8.6 %, respectively. The reported mean intra- and inter-assay coefficients of variation for sTNFR2 are 3.5 % and 4.1 %, respectively.

Study groups and nomenclature

Five groups were created in order to identify the effects of inhibition of IL-1 and TNF- α on healing time after a simple articular fracture. Group 1 (fracture with no treatment, denoted Fx) acted as a fractured control group, where no treatment of any kind was given post-fracture. Group 2 acted as the vehicle control, with intra-articular delivery of xELP[PBS]. Group 3 received intra-articular injection of the IL-1 receptor antagonist (anakinra) with the xELP[IL-1Ra] depot. Group 4 received intra-articular injection of soluble TNF receptor II (etanercept) with the xELP[sTNFR2] depot. Group 5 combined the inhibition of IL-1 and TNF- α by giving IL-1Ra and sTNFR2 together, denoted xELP[IL-1Ra+sTNFR2].

Animals

All animal procedures were performed in accordance with protocols approved by the Duke University Institutional Animal Care and Use Committee (IACUC) and the United States Army Medical Department Medical Research and Materiel Command Animal Care and Use Review Office (USAMRMC ACURO). Male C57BL/6J mice ($n = 77$, Charles River Laboratories, Wilmington, MA, USA) were obtained at 11 weeks of age. Mice were housed until 16 weeks of age. Five groups were then designated, with all groups subjected to a closed articular fracture of the left knee, as described previously (Furman *et al.*, 2007). Groups 1, 2, and 3 each contained 16 mice, group 4 contained 12 mice, and group 5 contained 14 mice. 16 additional C57BL/6J mice were obtained and sacrificed at 4 weeks and 8 weeks to serve as age-matched controls for the biomarker analyses.

Articular fracture protocol

Closed articular fractures were created using a previously described murine articular fracture model (Furman *et al.*,

2007). Each mouse was sedated (pentobarbital, IP 60 mg/kg) and placed into a custom cradle that positioned the left hind limb at neutral (90° flexion). This cradle was then placed onto the ElectroForce™ 3200 (EnduraTEC Systems, Minnetonka, MN, USA). A preload of -10 N was then applied to the tibia using a custom polished stainless-steel wedge-shaped indenter. The tibia was then loaded in compression at a rate of 20 N/s (55 N maximum) using a -3.2 mm displacement limit to create a simple fracture. Groups 2-5 then received their designated treatment immediately after fracture. For intra-articular injections, Hamilton syringes were used with 27G needles under sterile conditions.

Collection of materials at sacrifice

Mice from each group were sacrificed at 4 weeks ($n = 6-8$) and 8 weeks ($n = 6-8$) post-fracture. At the time of sacrifice, serum and synovial fluid from each limb were collected from each mouse and stored for use in biomarker analysis, as described previously (Seifer *et al.*, 2008). Both hind limbs from each mouse were harvested and fixed at 90° flexion in 10 % neutral buffered formalin for 5 d, then transferred to 70 % ethanol.

MicroCT assessment

A scan of each limb was taken using micro-computed tomography (MicroCT 40, Scanco Medical AG, Bassersdorf, Switzerland) to perform bone morphology analysis. A global threshold procedure enabled the separation of calcified tissues and soft tissues, which allowed for analysis of specific landmarks in the knee. Three regions are of interest when identifying the effects of PTA: the cancellous bone fraction of the femoral condyles, the tibial plateaus, and the metaphyseal region of the tibia. The parameters that were evaluated include bone volume, bone density, cancellous bone fraction (bone volume/total volume), trabeculae number, trabeculae thickness, and trabeculae spacing. We subtracted the value of the contralateral control limb from the fractured limb to get the resultant change between limbs as a result of fracture for all measured values.

Histological processing of limbs

Immediately following scanning, hind limbs were prepared for histology. Decalcification of the limbs was done using Cal-Ex Decalcification Solution (Fisher Scientific, Pittsburgh, PA, USA) for 4-5 d. Limbs were then dehydrated, infiltrated, and paraffin-embedded using the Leica ASP300S Tissue Processor (Leica Microsystems, Buffalo Grove, IL, USA).

Articular cartilage assessment

Articular cartilage pathology was evaluated using histological analysis and semi-quantitative grading. Sequential sections (8 μm) of fixed limbs in the coronal plane were obtained and stained using Safranin-O (Sigma-Aldrich, St. Louis, MO, USA), Harris Hematoxylin (Poly Scientific, Bay Shore, NY, USA), and Fast Green (Electron Microscopy Sciences, Hatfield, PA, USA). A modified Mankin histological scoring system was used by

three blinded graders to evaluate cartilage pathology and degenerative changes (Furman *et al.*, 2007). The lateral femur, lateral tibia, medial femur, and medial tibia were each graded separately. Parameters examined include subchondral bone thickness, articular cartilage structure, tidemark duplication, Safranin-O staining, fibrocartilage, chondrocyte clones, and hypertrophic chondrocytes. A total modified Mankin score representing the state of the joint cartilage was obtained by summing the individual location scores with a maximum score of 120 possible for each joint.

Synovial inflammation assessment

Synovial pathology was examined using histological analysis and semi-quantitative grading. Sequential sections of fixed limbs were stained using Harris Hematoxylin and Eosin (Poly Scientific). A modified synovial histological scoring system was used by three blinded graders to evaluate synovitis in each joint (Lewis *et al.*, 2013). The synovial insertion of the lateral femur, lateral tibia, medial femur, and medial tibia were each graded separately. Parameters examined include synovial lining thickness and cellular density in the synovial stroma. A total modified synovitis score per side was obtained by summing the individual location scores on the lateral side and the medial side with a maximum score of 12 possible for each side of the joint.

Serum and synovial fluid biomarker assays

Serum and synovial fluid collected at sacrifice were used to perform biomarker analyses. An age-matched control group, which received no fractures or treatments, served as a biomarker control group. Serum ELISAs for interleukin 6 (IL-6), macrophage-derived chemokine (MDC), thymus and activation-regulated chemokine (TARC), matrix metalloproteinase-3 (MMP-3) (R&D Systems), cartilage oligomeric matrix protein (COMP) (MD Bioproducts, St. Paul, MN, USA), hyaluronic acid (HA) (Corgenix, Broomfield, CO, USA), transforming growth factor-β₁ (TGF-β₁) (BioLegend, San Diego, CA, USA) and synovial fluid assays for IL-6 (R&D Systems) and COMP were performed according to the manufacturers' instructions. For the purposes of statistical analyses, in each of the assays, ½ the lower limit of detection (LLOD), or minimum detectable dose, was substituted for any value that was determined to be below the limit of detection. Serum and synovial fluid levels of mouse IL-6 were quantified by sandwich ELISA utilizing 50 μL of undiluted serum or synovial fluid diluted 1:5, requiring 10 μL of sample. The reported mean intra- and inter-assay coefficients of variation of the assay were 4.7 % and 7.5 %, respectively, and the minimum detectable dose was reported to be 1.6 pg/mL. For serum IL-6, 38 % of the samples (34 of 90) were below the level of detection and for synovial fluid, 61 % of the samples were below the level of detection. Serum levels of MDC were quantified by sandwich ELISA utilizing 20 μL of sample in a 3-fold dilution. The reported mean intra- and inter-assay coefficients of variation of the assay were 5.4 % and 7.2 %, respectively, and the minimum detectable dose was reported to be 1.2 pg/mL. Values were obtained for all samples analyzed. Serum levels of

TARC were quantified by sandwich ELISA utilizing 50 μ L of undiluted sample. The reported mean intra- and inter-assay coefficients of variation of the assay were 3.4 % and 6.7 %, respectively, and the minimum detectable dose was reported to be 5 pg/mL. Values were obtained for all samples analyzed. Serum levels of MMP-3 were assessed by sandwich ELISA at a dilution of 1:20. The reported mean intra- and inter-assay coefficients of variation of the assay were 3.0 % and 7.6 %, respectively, and the minimum detectable dose was reported to be 0.019 ng/mL. Values were obtained for all samples analyzed. Serum and synovial fluid levels of mouse COMP were quantified by competitive ELISA requiring a 1:10 dilution of serum and a 1:5 dilution of synovial fluid. The reported mean intra- and inter-assay coefficients of variation of the assay were 9.4 % and 6.5 %, respectively, and the minimum detectable dose was reported to be 0.2 U/L. Values were obtained for all samples analyzed. Serum levels of hyaluronic acid were quantified by sandwich ELISA utilizing 12 μ L of sample. Information on the coefficients of variation of the assay were not provided by the manufacturer, however, three controls (low, moderate, and high) were provided and all ran within the acceptable range. Values were obtained for all samples analyzed. Two different sandwich ELISAs were employed for the determination of Free Active TGF- β_1 and Total TGF- β_1 . Samples were run undiluted for Free Active TGF- β_1 and diluted 1000-fold for Total TGF- β_1 , as recommended by the manufacturer. The minimal detectable concentration of Free Active TGF- β_1 was reported as 2.3 pg/mL and 3.5 pg/mL for Total TGF- β_1 . The intra- and inter-assay CVs were 9.1 % and 8.0 %, respectively, for Free Active TGF- β_1 and 3.6 % and 6.6 %, respectively, for Total TGF- β_1 . Values were obtained for all samples.

Statistical analysis

All analyses were conducted using STATISTICA (v.7, StatSoft, Inc., Tulsa, OK, USA) with significance reported at the 95 % confidence level. Data were examined using repeated measures to examine differences between the control and experimental limb, along with the reduction of PTA symptoms among the different treatment types and times. For data that were normally distributed, parametric analysis was performed; otherwise, non-parametric analysis was performed.

For the microCT analysis, a multifactorial ANOVA was performed with the contralateral control limb and fractured limb as the repeated measure. Treatment groups and time points served as the dependent variables. A Tukey honestly significant difference (HSD) *post-hoc* test was performed to determine significance between treatment groups and time points.

For the Mankin and synovitis scores, non-parametric analysis was performed. A Wilcoxon matched pairs test was used to report significance between the contralateral control limb and the fractured limb. A Kruskal-Wallis ANOVA was used to compare individual groups against each other at each time point. A Mann-Whitney U test was performed to determine statistical differences within a group at 4 and 8 weeks.

For the biomarker data, non-parametric analysis was performed. A Kruskal-Wallis ANOVA was used to compare

individual groups at each time point, and a Mann-Whitney U test was performed to determine statistical differences within groups at 4 and 8 weeks.

Results

Release of drug from xELP *in vitro* showed sustained release of IL-1Ra and sTNFR_{II} over 7 days

Drugs were loaded into cross-linked ELP depots (xELP) by rehydrating the lyophilized xELP in drug-containing solutions (Fig. 1a). Under these conditions and for this ELP sequence, the temperature-induced transition is retained at 37 °C and is observed as a change in opacity of the drug-loaded xELP construct (Fig. 1b). *In vitro* experiments in 10 % serum-containing media were conducted in order to determine the drug release profile from the cross-linked xELP depots. The 10 % serum-containing media and 37 °C culture condition was used to mimic the conditions present in synovial fluid. Over a period of 7 d, the release profiles of both xELP[IL-1Ra] (Fig. 1c) and xELP[sTNFR_{II}] (Fig. 1d) were similar, with a release of drug that plateaued at day 2 and day 3, respectively. With xELP depot formation at 37 °C, the majority of the drug was encapsulated. However, there was an initial release of drug from the xELP depot that likely reflects drug that was not fully encapsulated by the transitioning xELP. The *in vitro* release profiles of both IL-1Ra and sTNFR_{II} indicated that the xELP depots successfully released these drugs over an extended 7-day period.

Release of drug from xELP *in vivo* showed sustained release of IL-1Ra and sTNFR_{II} over 5 days

Serial blood draws were taken from the mice throughout the study and processed for serum collection; due to the small volume, mice from each treatment group at each time point were pooled to determine the serum levels of human IL-1Ra (hIL-1Ra) and sTNFR_{II} (hsTNFR_{II}) in the mice following intra-articular drug delivery. As expected, the levels of hIL-1Ra were undetectable in the fracture only with no treatment (Fx) group, and in the group receiving depots loaded with PBS only (xELP[PBS]). The serum of the xELP[IL-1Ra] group had measurable levels of hIL-1Ra on day 1 of ~ 5 ng/mL, and still had detectable levels at day 5 (Fig. 2a). In the xELP[IL-1Ra+sTNFR_{II}] group, there was a detectable serum level of hIL-1Ra of over 30 ng/mL on day 1, which then fell to an undetectable level by day 5. As expected, levels of hsTNFR_{II} were undetectable in the Fx group and the xELP[PBS] group (Fig. 2b). The xELP[sTNFR_{II}] group had measurable serum levels of hsTNFR_{II} of over 1,100 ng/mL on day 1, which fell to 300 ng/mL by day 5 and was undetectable by day 14. In the combination therapy, serum hsTNFR_{II} was greater than 550 ng/mL on day 1, dropped to ~ 450 ng/mL on day 5, and was undetectable by day 14. The *in vivo* release profiles indicated that the single drug therapies remained in the serum for at least 5 d after an intra-articular injection, and the combination therapy drugs remained in the serum for 1-5 d after an intra-articular injection, demonstrating that the xELP depots successfully released the drugs over an extended period of 5 d.

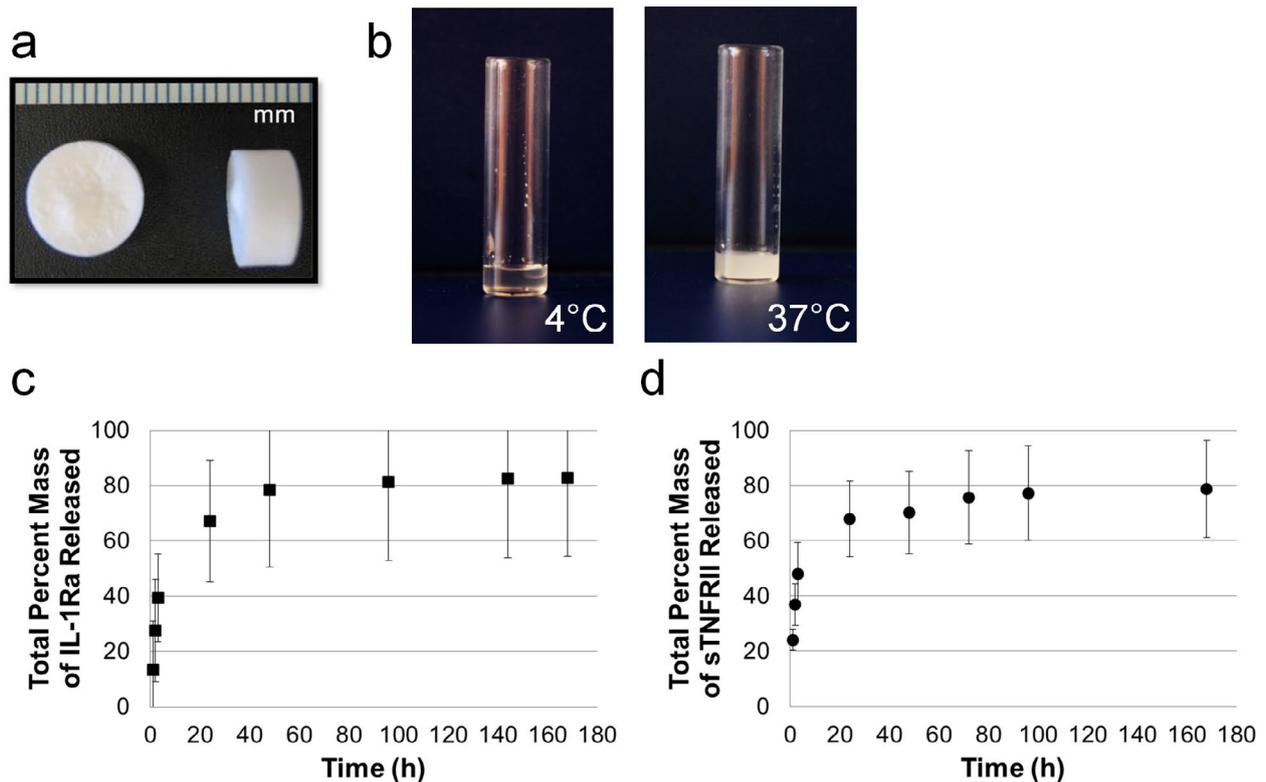


Fig. 1. Results of *in vitro* drug release studies. (a) Close-up image of lyophilized xELP constructs. (b) Drug rehydrated xELP constructs at 4 °C (left) and at 37 °C (right). Change in opacity of the xELP at the transition-induced temperature of 37 °C is seen by comparing the left and right pictures. (c) Drug release of IL-1Ra from xELP in 10 % serum-containing medium over 180 h. Mean \pm standard deviation reported ($n = 4$). (d) Drug release of sTNFRII from xELP in 10 % serum-containing medium over 180 h. Mean \pm standard deviation reported ($n = 4$).

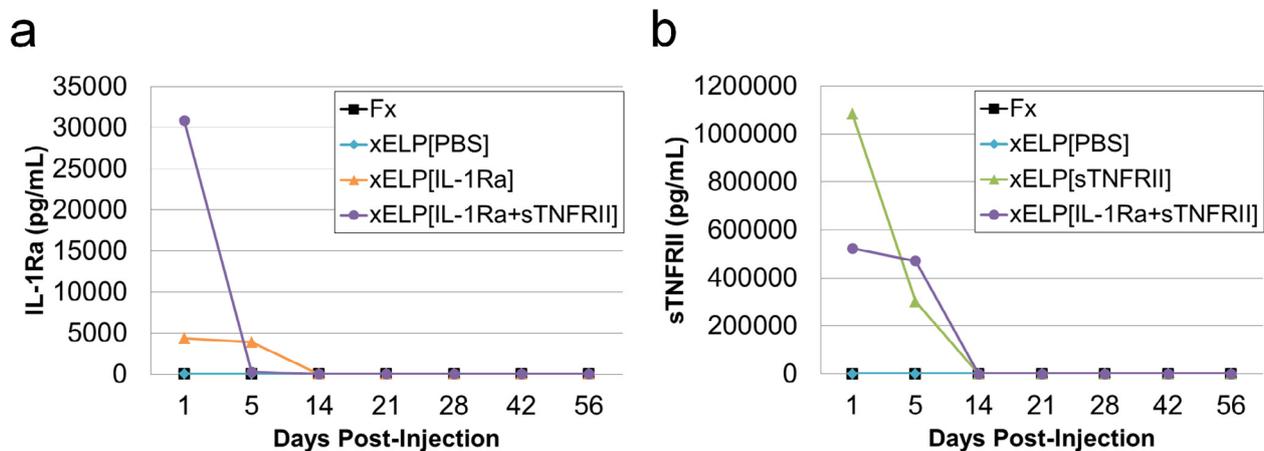


Fig. 2. Results of *in vivo* drug release studies. (a) Drug release of IL-1Ra from xELP in mouse serum as measured by ELISA. Fx and xELP[PBS] were run as background controls for ELISA. Data are mean of samples in triplicate ($n = 6-11$ per data point). (b) Drug release of sTNFRII from xELP in mouse serum as measured by ELISA. Fx and xELP[PBS] were run as background controls for ELISA. Data are mean of samples in triplicate ($n = 6-11$ per data point).

Histological assessment of joint tissues showed that xELP[IL-1Ra] reduced articular cartilage degeneration

Articular cartilage degeneration was quantified from histological sections using the Mankin score of the contralateral control limb and the fractured limb for each mouse. At 4 weeks (Fig. 3a), there was no significant increase in Mankin scores between the fractured limb and

the contralateral control limb in the fracture only with no treatment (Fx) group ($p = 0.09$), the xELP[PBS] group ($p = 0.16$), the xELP[IL-1Ra] group ($p = 0.67$) or the xELP[IL-1Ra+sTNFRII] group ($p = 0.18$); however, in the xELP[sTNFRII] group at 4 weeks, there was a significant increase in Mankin score between the fractured limb and the contralateral control limb ($p = 0.04$). By 8 weeks (Fig. 3b) there were significantly greater Mankin scores in

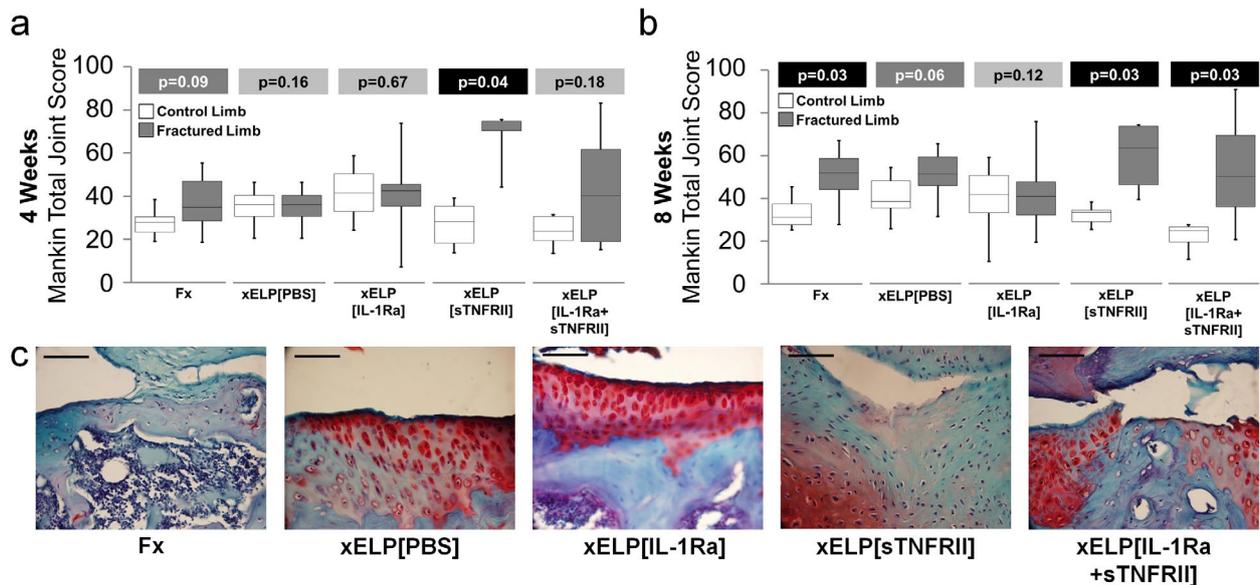


Fig. 3. Assessment of articular cartilage degeneration. (a) Modified Mankin total joint score for limbs at 4 weeks. p values denote significance between control and fractured limb using Wilcoxon Matched Pairs test. Median values and 25th-75th quartile range reported ($n = 6-8$ per group). (b) Modified Mankin total joint score for limbs at 8 weeks. p values denote significance between control and fractured limb using Wilcoxon Matched Pairs test. Median values and 25th-75th quartile range reported ($n = 6-8$ per group). (c) Representative histological pictures of articular cartilage on the lateral tibial plateau taken at 40 \times magnification for each group with Safranin-O/Fast Green staining at 8 weeks. Scale bar indicates 5 μ m.

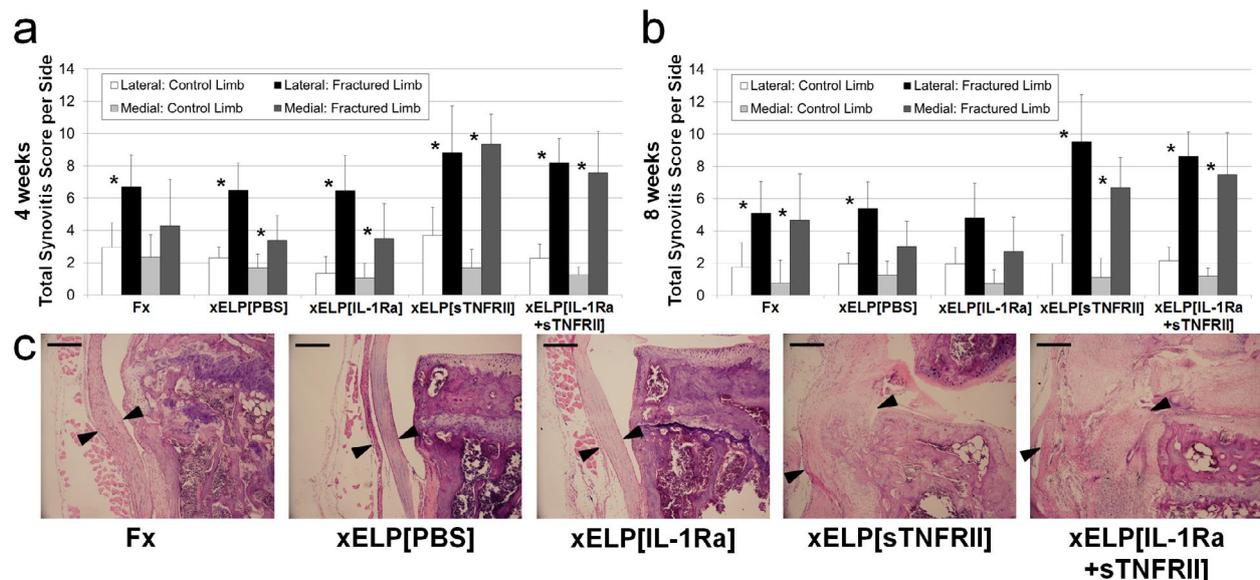


Fig. 4. Assessment of synovial inflammation. (a) Modified Krenn joint score for medial and lateral sides of each limb at 4 weeks. * denotes $p < 0.05$ between control and fractured limb using Wilcoxon Matched Pairs test. Mean \pm standard deviation reported ($n = 5-8$ per group). (b) Modified Krenn joint score for medial and lateral sides of each limb at 8 weeks. * denotes $p < 0.05$ between control and fractured limb using Wilcoxon Matched Pairs test. Mean \pm standard deviation reported ($n = 5-8$ per group). (c) Representative histological pictures of synovium (black arrows) in the lateral tibial plateau quadrant of knee joints at 8 weeks using Hematoxylin and Eosin stained sections. Scale bar indicates 10 μ m.

the fractured limb compared to the contralateral control limb for the Fx group ($p = 0.03$), the xELP[sTNFRII] group ($p = 0.03$), and the xELP[IL-1Ra+sTNFRII] group ($p = 0.03$). There was also a trend of increased Mankin scores in the fractured limb of the xELP[PBS] group ($p = 0.06$) at 8 weeks. In contrast, there was no increase in Mankin score in the fracture limb of the xELP[IL-1Ra]

group ($p = 0.12$) at 8 weeks. Histology of the lateral tibia at 8 weeks showed a severe loss of proteoglycan staining and subsequent fibrocartilage healing in the fractured limb of the Fx group (Fig. 3c). In the xELP[PBS] group, there was evidence of surface fibrillation in the articular cartilage, while in the xELP[IL-1Ra] group, the fractured limb demonstrated only minimal structural changes. In

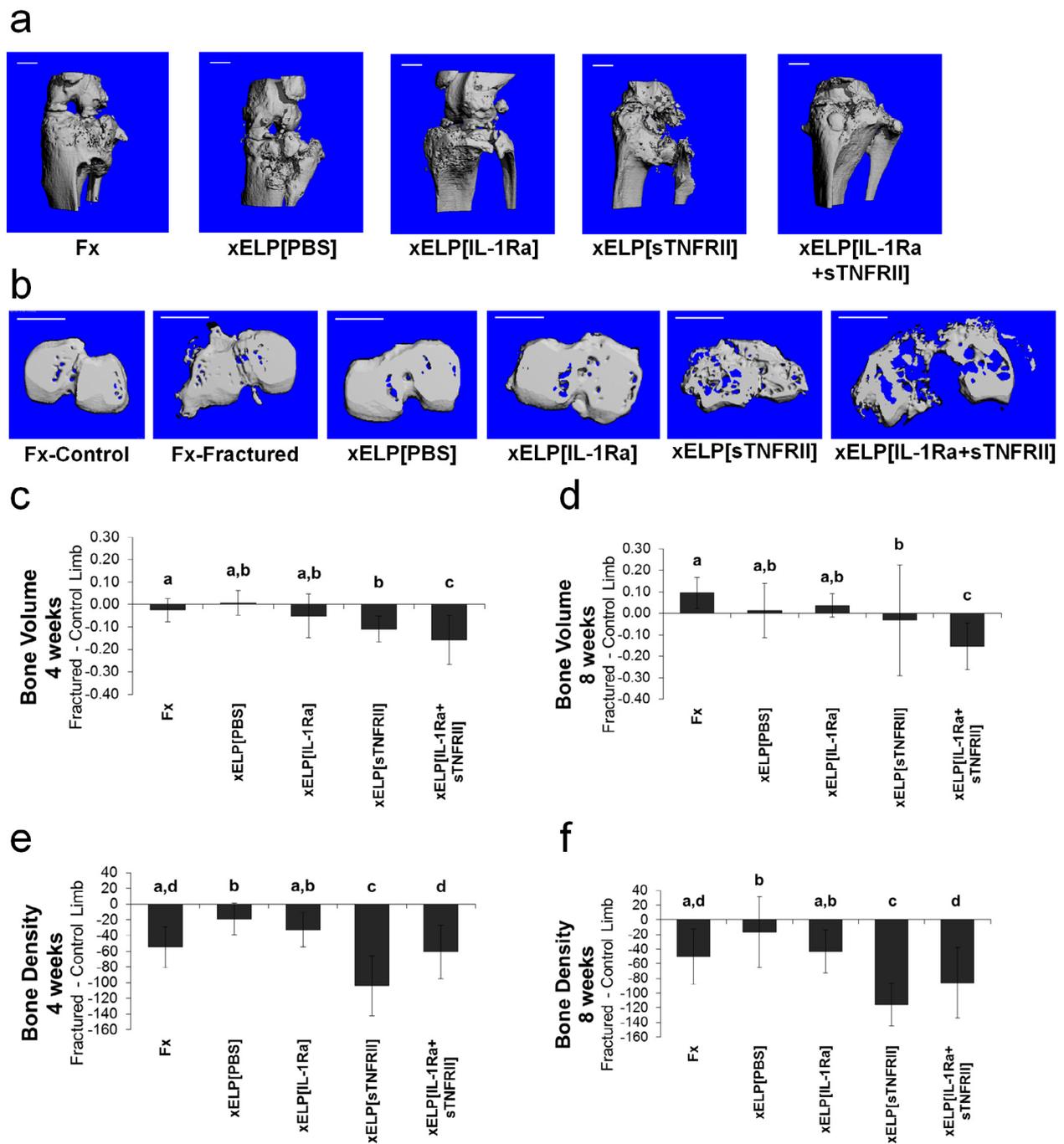


Fig. 5. Assessment of bone morphological changes. **(a)** Representative microCT images of the fractured limb from each group at 8 weeks. Fractures are present in each image in the lateral tibial plateau. Scale bar indicates 1 mm. **(b)** Representative microCT images of the tibial plateau of a control limb (first picture) and fractured limbs (pictures 2-6) from each group at 8 weeks. Scale bar indicates 1 mm. **(c)** Bone volume data shown at 4 weeks for each group. Tukey HSD *post-hoc* test performed; groups that do not share letters are significantly different from each other ($p < 0.05$). Mean \pm standard deviation reported ($n = 5-8$ per group). **(d)** Bone volume data shown at 8 weeks for each group. Tukey HSD *post-hoc* test performed; groups that do not share letters are significantly different from each other ($p < 0.05$). Mean \pm standard deviation reported ($n = 5-8$ per group). **(e)** Bone density data shown at 4 weeks for each group. Tukey HSD *post-hoc* test performed; groups that do not share letters are significantly different from each other ($p < 0.05$). Mean \pm standard deviation reported ($n = 5-8$ per group). **(f)** Bone density data shown at 8 weeks for each group. Tukey HSD *post-hoc* test performed; groups that do not share letters are significantly different from each other ($p < 0.05$). Mean \pm standard deviation reported ($n = 5-8$ per group).

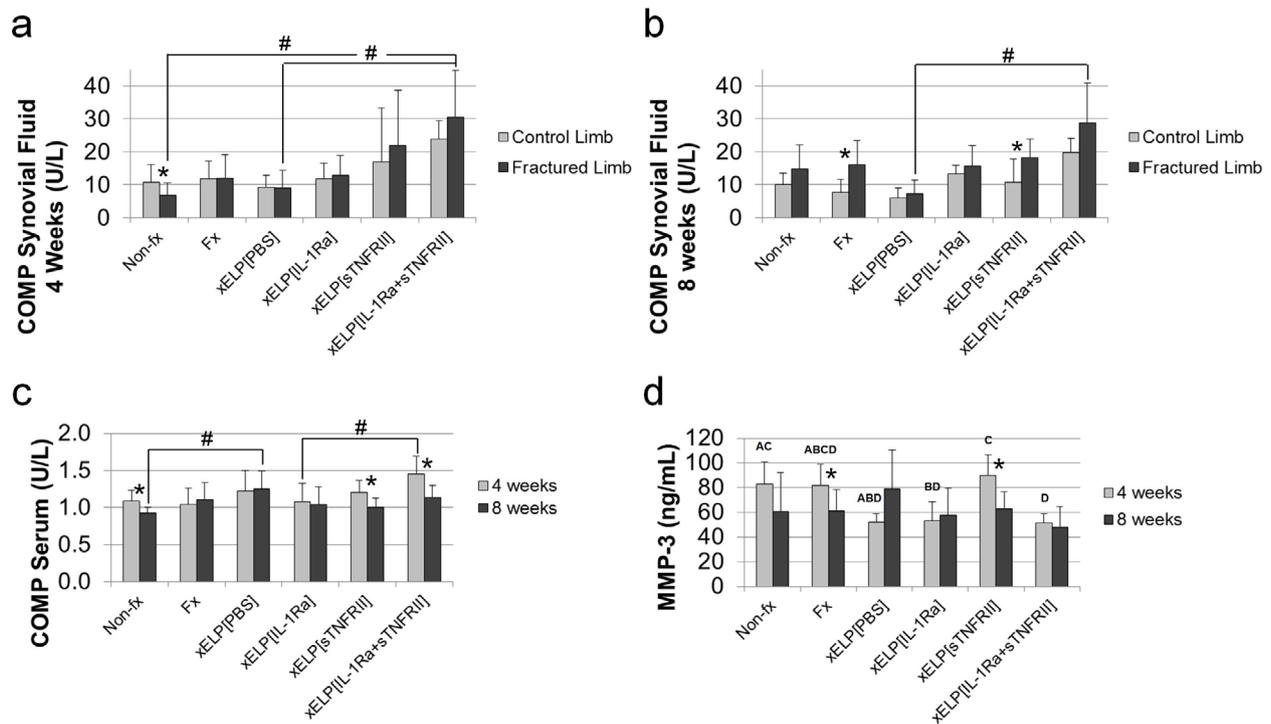


Fig. 6. Assessment of serum and synovial fluid biomarkers. **(a)** Levels of cartilage oligomeric matrix protein (COMP) in synovial fluid in the limbs at 4 weeks. Mean \pm standard deviation reported ($n = 5-8$ per group). # denotes $p < 0.05$ between groups using Kruskal-Wallis ANOVA. * denotes $p < 0.05$ between fractured and control limb using Wilcoxon Matched Pairs test. **(b)** Levels of COMP in synovial fluid in the limbs at 8 weeks. Mean \pm standard deviation reported ($n = 5-8$ per group). # denotes $p < 0.05$ between groups using Kruskal-Wallis ANOVA. * denotes $p < 0.05$ between fractured and control limb using Wilcoxon Matched Pairs test. **(c)** Levels of COMP in serum for each group at 4 and 8 weeks. Mean \pm standard deviation reported ($n = 5-8$ per group). # denotes $p < 0.05$ between groups using Kruskal-Wallis ANOVA. * denotes $p < 0.05$ between 4 and 8 weeks using Kruskal-Wallis ANOVA test. **(d)** Levels of matrix metalloproteinase-3 (MMP-3) in serum for each group at 4 and 8 weeks. Mean \pm standard deviation reported ($n = 5-8$ per group). * denotes $p < 0.05$ between 4 and 8 weeks using Kruskal-Wallis ANOVA. Groups that do not share letters are significantly different ($p < 0.05$) at 4 weeks by Kruskal-Wallis ANOVA.

contrast, the xELP[sTNFRII] limb showed an outgrowth of fibrocartilage at the site of fracture which made it difficult to distinguish the original margins of the articular cartilage surface. A similar phenomenon was present in the xELP[IL-1Ra+sTNFRII] limb, where there was little evidence of fracture healing save for some strands of fibrocartilage seen near the boundary of the growth plate.

Histological assessment showed that xELP[IL-1Ra] reduced the severity of synovitis

The degree of synovial inflammation was assessed from histological sections using a synovitis score of the contralateral control limb and the fractured limb for each mouse. At 4 weeks (Fig. 4a), all 5 groups showed a significant difference between the contralateral control limb and the fractured limb on the lateral side of the joint, where the fracture was typically located. On the medial side of the joint, the fracture only with no treatment (Fx) group was the only group that did not show a significant increase in synovitis between the fractured and contralateral control limb ($p = 0.21$); the other 4 groups had increased synovitis in the fractured limb ($p < 0.05$). However, at 8 weeks (Fig. 4b), both lateral and medial sides of the joint showed increased synovitis in the fractured limb for the

Fx group ($p = 0.04$, $p = 0.01$), the xELP[sTNFRII] group ($p = 0.03$, $p = 0.03$), and the xELP[IL-1Ra+sTNFRII] group ($p = 0.03$, $p = 0.03$). From the representative histological pictures of the medial tibia taken at 8 weeks (Fig. 4c), the fractured limb in the Fx group demonstrated increased inflammation in the synovial attachment to the tibial plateau. The xELP[PBS] group and the xELP[IL-1Ra] group had minimal synovial inflammation and did not show a significant difference between limbs in total synovitis score on the medial side. Both the xELP[sTNFRII] group and the xELP[IL-1Ra+sTNFRII] group showed inflamed synovium, with dramatically increased cellular infiltration. From the synovitis grading and images, we concluded that both the xELP[PBS] and xELP[IL-1Ra] treatments provided a reduction in synovial inflammation on the medial side, and that treatment with xELP[IL-1Ra] also provided a benefit on the lateral side of the joint.

MicroCT assessment of bone properties showed a trend towards increased bone volume, but decreased bone density at 8 weeks

Bone morphological changes were assessed using micro-computed tomography (microCT) in the contralateral control and fractured limbs. Fractures were confirmed

Table 1. Biomarker levels in mouse serum and synovial fluid at 4 and 8 weeks.

<i>SERUM* 4 weeks</i>	Non-fx	Fx	ELP-PBS	ELP-IL-1Ra	ELP-sTNFRII	ELP-IL-1Ra+sTNFRII	Kruskal-Wallis <i>p</i> (groups)†
HA (pg/mL)	3.50e5±0.14e5	3.31e5±0.88e5	3.56e5±0.12e5	4.18e5±0.14e5	3.35e5±0.89e5	2.89e5±0.49e5	none
IL-6 (pg/mL)	117.00±91.51 ^A	34.29±34.55 ^{ABC}	8.81±13.96 ^{BC}	16.03±14.58 ^{ABC}	28.05±22.47 ^{ABC}	2.73±5.59 ^{BC}	<i>p</i> < 0.002
MDC (pg/mL)	224.73±29.78	212.23±43.46	207.50±12.95	213.46±22.93	189.98±12.91	226.89±36.15	none
TARC (pg/mL)	101.60±14.39 ^{AB}	82.38±17.91 ^A	102.65±15.50 ^{AB}	93.00±8.42 ^{AB}	93.72±7.93 ^{AB}	108.06±12.46 ^A	<i>p</i> = 0.0095
TGF-β ₁ Free Active (pg/mL)	181.34±63.76	193.81±52.03	143.23±33.98	157.56±38.74	196.02±33.48	184.23±44.27	none
TGF-β ₁ Total (pg/mL)	62692.25±9531.97 ^{AB}	71449.75±10323.00 ^{AB}	56167.29±5723.62 ^A	64542.13±6538.26 ^{AB}	76986.33±15707.00 ^B	63363.00±8615.71 ^{AB}	<i>p</i> = 0.0230
TGF-β ₁ Free Active/Total	0.0029±0.0010	0.0027±0.0006	0.0025±0.0006	0.0025±0.0007	0.0028±0.0012	0.0030±0.0009	none
<i>SERUM* 8 weeks</i>	Non-fx	Fx	ELP-PBS	ELP-IL-1Ra	ELP-sTNFRII	ELP-IL-1Ra+sTNFRII	Kruskal-Wallis <i>p</i> (groups)†
HA (pg/mL)	4.41e5±0.11e5	4.87e5±0.32e5	4.29e5±0.14e5	3.71e5±0.11e5	3.15e5±0.46e5	3.81e5±0.15e5	none
IL-6 (pg/mL)	79.70±95.10	63.52±88.89	40.66±52.27	7.15±11.43	10.08±11.51	2.09±3.59	none
MDC (pg/mL)	193.27±21.60	213.38±16.79	204.06±44.83	193.89±28.03	206.93±45.17	232.76±26.58	none
TARC (pg/mL)	101.39±15.61	101.59±8.18	104.41±19.38	104.08±16.89	95.10±24.30	106.88±12.03	none
TGF-β ₁ Free Active (pg/mL)	198.20±64.79 ^{AB}	175.55±17.24 ^{AB}	113.72±31.17 ^A	161.51±64.68 ^{AB}	174.44±52.46 ^{AB}	256.71±124.00 ^B	<i>p</i> = 0.0086
TGF-β ₁ Total (pg/mL)	60755.63±14857.95 ^{ABC}	72337.88±7516.89 ^{BC}	51357.75±5738.49 ^A	59918.53±9272.15 ^{ABC}	67391.83±8336.70 ^{ABC}	72954.14±5864.54 ^{BC}	<i>p</i> < 0.005
TGF-β ₁ Free Active/Total	0.0034±0.0013	0.0024±0.0040	0.0023±0.0007	0.0027±0.0010	0.0026±0.0008	0.0035±0.0017	none
<i>SYNOVIAL FLUID* 4 weeks</i>	Non-fx	Fx	ELP-PBS	ELP-IL-1Ra	ELP-sTNFRII	ELP-IL-1Ra+sTNFRII	Kruskal-Wallis <i>p</i> (groups)†
IL-6 R (pg/mL)	3.29±9.08	7.91±14.55	0.08±0.00	0.08±0.00	6.97±16.87	0.08±0.00	none
IL-6 L (pg/mL)	9.49±18.45	8.41±15.43	2.43±4.38	0.08±0.00	3.54±8.47	0.08±0.00	none
<i>SYNOVIAL FLUID* 8 weeks</i>	Non-fx	Fx	ELP-PBS	ELP-IL-1Ra	ELP-sTNFRII	ELP-IL-1Ra+sTNFRII	Kruskal-Wallis <i>p</i> (groups)†
IL-6 R (pg/mL)	3.15±8.69	8.09±14.85	1.36±3.62	0.08±0.00	7.27±11.14	0.08±0.00	none
IL-6 L (pg/mL)	7.10±13.24	4.83±9.19	2.76±5.19	1.25±3.30	22.33±18.07	0.08±0.00	none

*Data are presented as mean ± standard deviation (*n* = 5-8 per group).

†Groups with different letters are significant from each other as indicated by the Kruskal-Wallis *p* value.

using microCT images of the entire knee joint (Fig. 5a). In the epiphysis of the tibial plateau, which included the site of articular fracture, morphologic parameters were quantified in the fractured limb and normalized to the contralateral control limb. Representative images of each group showed the effects of the fracture on bone architecture in the tibial plateau (Fig. 5b). At 4 weeks post-fracture (Fig. 5c), most treatment groups demonstrated a decrease in bone volume. However, the xELP[sTNFRII] group had a decrease in bone volume that was significant compared to the fracture only with no treatment (Fx) group (*p* < 0.05), and the xELP[IL-1Ra+sTNFRII] group had a decrease in bone volume that was significant compared to the other 4 groups (*p* < 0.05). Likewise, at 8 weeks (Fig. 5d), most treatment groups demonstrated an increase in bone volume. However, the xELP[sTNFRII] had a decrease in bone volume that was significant compared to the Fx group (*p* < 0.05), and the xELP[IL-1Ra+sTNFRII] group had a decrease in bone volume that was significant compared to the other 4 groups (*p* < 0.05).

All groups demonstrated a decrease in bone density in the epiphysis of the tibial plateau at 4 weeks (Fig. 5e) and at 8 weeks (Fig. 5f). The xELP[PBS] group was significantly different than the Fx group (*p* < 0.05), and the xELP[sTNFRII] group was significantly different compared to the other 4 groups (*p* < 0.05). These results indicated that over 8 weeks, bone density was not increasing as a result of fracture healing. The increase in bone volume that was seen in the Fx, xELP[PBS], and xELP[IL-1Ra] groups at 8 weeks did not contribute to an increase in bone density in the same groups at 8 weeks.

Quantification of PTA-related biomarkers

Synovial fluid COMP levels in the fractured limb were significantly different between the xELP[PBS]

and xELP[IL-1Ra+sTNFRII] groups at both 4 weeks (*p* = 0.0103, Fig. 6a) and 8 weeks (*p* = 0.0004, Fig. 6b). COMP levels in the xELP[IL-1Ra+sTNFRII] group were also significantly different compared to the Non-fx group at 4 weeks (*p* = 0.0009). Serum COMP levels (Fig. 6c) were significantly lower in the xELP[IL-1Ra] group compared with the xELP[IL-1Ra+sTNFRII] group at 4 weeks (*p* = 0.031) and similar to levels in the non-Fx group. At 8 weeks, serum COMP levels in the Non-fx group were significantly lower compared to the xELP[PBS] group (*p* = 0.035). Serum MMP-3 levels decreased compared to Non-fx controls when IL-1Ra was delivered either alone (xELP[IL-1Ra], *p* = 0.041) or in combination with sTNFRII (xELP[IL-1Ra+sTNFRII], *p* = 0.034) at 4 weeks (Fig. 6d). No differences were seen at 8 weeks. Serum IL-6 (Table 1) showed a significant difference at 4 weeks between the Non-fx group and the xELP[PBS] group (*p* = 0.015) and between the Non-fx group and the xELP[IL-1Ra+sTNFRII] group (*p* = 0.0025), but no differences at 8 weeks. Serum levels of HA and MDC (Table 1) did not show any differences between groups at 4 or 8 weeks. Serum levels of TARC (Table 1) showed a significant difference between the xELP[PBS] group and the xELP[sTNFRII] group at 4 weeks (*p* = 0.023), but did not show differences at 8 weeks. Free active TGF-β₁ in serum (Table 1) showed few differences among groups with the exception of higher concentrations in the xELP[IL-1Ra+sTNFRII] group compared to the xELP[PBS] group at 8 weeks (*p* = 0.0086).

Correlations between biomarkers, histology, and bone morphology

Despite the limited amount of differences between groups at both 4 and 8 weeks, there were interesting correlations between the biomarkers and Mankin scores, synovitis

scores, and bone properties. For example, the synovial fluid COMP levels in the fractured limb positively correlated with the synovitis score of the fractured limb at both 4 and 8 weeks ($r_s = 0.69$, $p = 0.00001$; $r_s = 0.41$, $p = 0.0094$), and the IL-6 serum levels negatively correlated with the synovitis score of the fractured limb at 4 weeks ($r_s = -0.50$, $p = 0.0016$). Biomarkers also correlated with other biomarkers; for example, the fractured limb synovial fluid COMP levels positively correlated with the serum COMP levels at 4 weeks ($r_s = 0.41$, $p = 0.0055$). IL-6 serum positively correlated with serum MMP-3 levels at 4 weeks ($r_s = 0.39$, $p = 0.0093$). Synovial fluid COMP levels from the fractured limb did not correlate with serum MMP-3 levels at 4 weeks; however, synovial fluid COMP negatively correlated with the serum MMP-3 levels ($r_s = -0.34$, $p = 0.020$) at 8 weeks. Serum MMP-3 also positively correlated with the serum COMP ($r_s = 0.30$, $p = 0.048$) and negatively correlated with serum MDC at 8 weeks ($r_s = -0.34$, $p = 0.020$).

Discussion

This study demonstrated that sustained release of IL-1Ra in the joint space reduced the severity of arthritic changes in both cartilage and synovium following intra-articular fracture. In contrast, the sustained delivery of sTNFR_{II} alone in the joint space, or in combination with IL-1Ra, resulted in detrimental bone, cartilage, and synovial changes. Importantly, we found that cross-linked ELPs provided for the entrapment of the protein drugs studied here, and for the sustained release observed *in vitro* and *in vivo* following intra-articular delivery. These findings demonstrate the potential of a beneficial therapeutic approach as an adjunct to surgical treatment for the prevention of PTA: the sustained intra-articular release of IL-1Ra significantly reduced the development of PTA following articular fracture when used in combination with cross-linked ELP as a thermally-sensitive drug depot.

Sustained administration of IL-1Ra reduced the level of cartilage degeneration in the joint and did not detrimentally affect bone healing. Other studies have shown similar results with the use of IL-1Ra; for instance, Lange and co-workers found that inactivating IL-1RI in mice did not affect long bone healing (Lange *et al.*, 2010). We did not observe detrimental effects on bone healing or bone morphological changes in the xELP[IL-1Ra] treatment group. The mice that received IL-1Ra exhibited a significantly smaller decrease in both bone volume and bone density compared to the sTNFR_{II} and IL-1Ra+sTNFR_{II} groups. These data support the conclusion that bone healing was not adversely affected by the presence of IL-1Ra, while the addition of TNFR_{II} *via* an intra-articular route did alter bone healing.

In our study, the Mankin scores of the IL-1Ra group were decreased compared to the sTNFR_{II} and IL-1Ra+sTNFR_{II} groups at both 4 and 8 weeks, providing evidence that IL-1Ra has some protective effect on articular cartilage after articular fracture compared to no treatment. Examination of the histologic sections also provided evidence of this protective effect; cartilage from the IL-1Ra group had the most intact structure and robust

staining compared to the other groups. The results suggest that the PBS group also showed reduced articular cartilage degeneration compared to the no treatment group, which may be due to a washing-out effect. The Mankin score of the PBS group showed a trend towards being significant at 8 weeks, unlike the IL-1Ra group. In a previous study, we showed that the administration of local saline reduced degenerative bone changes; however, it did not reduce cartilage degeneration or synovial inflammation (Furman *et al.*, 2014b). We also examined the synovium in our joints in order to grade the severity of synovitis as a result of articular fracture. Based on Mankin and synovitis scores, it is evident that degenerative changes occur globally throughout the joint in this mouse fracture model. At 8 weeks after fracture, the synovitis scores showed that the IL-1Ra and PBS groups were the only groups without a significant difference between the fractured limb and control on the medial side of the joint. However, the IL-1Ra group was the only group without a significant difference on the lateral side where the fracture occurred. The difference between limbs on the lateral side was significantly different for all the other groups. This finding shows that IL-1Ra not only reduced the level of cartilage degeneration, but also reduced the amount of synovitis in the joint. These findings are consistent with results of Caron and co-workers who showed that twice weekly injections of recombinant human IL-1Ra (rHuIL-1Ra) for four weeks demonstrated a chondroprotective effect against the development of OA after anterior cruciate ligament (ACL) transection in a canine model compared to saline control (Caron *et al.*, 1996), although the presence of an articular fracture in our model represents additional factors related to bone fracture and trauma that may lead to more rapid or severe degeneration. In a previous study, we showed that a single local injection of IL-1Ra given after articular fracture was able to reduce articular cartilage degeneration; however, systemic administration of IL-1Ra resulted in significant differences in Mankin scores between control and fractured limbs (Furman *et al.*, 2014b). These results underscore the utility of this model system for assessing PTA severity and for identifying new therapies for this disease process in humans.

In contrast to the effectiveness of IL-1Ra on the fractured joints in our model, sTNFR_{II} did not provide protection against PTA. Bone healing was inhibited in the presence of sTNFR_{II}, which may have contributed to the lack of protection against PTA changes. In our study, we did not observe the same effectiveness of sTNFR_{II} as seen in previous studies focused on RA (Bathon *et al.*, 2006; Bliddal *et al.*, 2006; Boesen *et al.*, 2008; Fiocco *et al.*, 2010; Johnsen *et al.*, 2006; Kavanaugh *et al.*, 2008; Krieckaert *et al.*, 2012; Moreland *et al.*, 1999; Roux *et al.*, 2011; Zwerina *et al.*, 2004). Similarly, Gerstenfeld and coworkers reported that a long bone fracture of the tibia in TNF receptor knockout mice resulted in non-union of the fracture (Gerstenfeld *et al.*, 2003). In our study, we also observed the detrimental effect of sTNFR_{II} on bone morphology. Compared to the fracture only group, the sTNFR_{II} and the IL-1Ra+sTNFR_{II} groups showed a significant decrease in bone volume, while the sTNFR_{II} group also showed a significant decrease in bone density. A similar result was

seen for articular cartilage degeneration; the sTNFR^{II} and IL-1Ra+sTNFR^{II} groups had a significantly higher Mankin score in the fractured limb compared to the contralateral control limb at 8 weeks. Elsaid and co-workers showed that blocking TNF- α in an ACL-transection model reduced the loss of GAG from articular cartilage (Elsaid *et al.*, 2009); however, in our case, the articular fracture plus the inhibition of TNF- α led to the irreversible damage of the articular cartilage, as reflected in the histological scores of the two sTNFR^{II} groups. The neutral or positive impact of TNF- α inhibition on soft tissue injury healing contrasted with the negative impact on bone fracture healing suggests that TNF- α is necessary for the fracture healing cascade (Fazzalari, 2011). This might indicate why the two groups that received sTNFR^{II} had such poor results compared to the other groups. We also noticed an increase in synovitis in the two groups that received sTNFR^{II}; in fact, synovial proliferation in these groups was so great that it was extremely difficult to identify where the synovium attached to the joints. Both the sTNFR^{II} and IL-1Ra+sTNFR^{II} groups showed a significant difference between the fractured limb and the contralateral control limb on the medial and lateral side at 8 weeks. The loss of TNF- α not only affected bone healing, but also increased the level of inflammation present in the joint after trauma. The timing of administration of sTNFR^{II} after injury may alter the effect of blocking TNF- α ; however, from our study, inhibiting TNF- α immediately after injury proved deleterious. Systemic delivery of both IL-1Ra and sTNFR^{II} in combination was also harmful; however, despite the increased risk of infection and contraindications (McEvoy, 2006) associated with administering both IL-1 and TNF inhibitors simultaneously, none of our mice showed signs of infection after an intra-articular injection of xELP[IL-1Ra+sTNFR^{II}].

An important finding of this study was the successful use of cross-linked ELP as a sustained-release drug depot for intra-articular anti-cytokine therapy. In previous studies, a fusion protein of ELP with IL-1Ra was shown to preserve the characteristics of both ELP and IL-1Ra and serve as a sustained-release delivery method for intra-articular therapy (Shamji *et al.*, 2007). Adams and co-workers demonstrated efficacy for this ELP-IL1Ra fusion protein against IL-1-induced arthritis in a rat model as compared to a single injection of low dose IL-1Ra (Allen *et al.*, 2010). Encapsulation of drug provides some significant advantages over drug fusions, namely, the ability to increase drug-loading ratios and to work with unmodified, native, and FDA-approved drugs. Adams and co-workers also demonstrated proof-of-concept for cross-linked ELPs to encapsulate antibiotics, retain the ELP thermal transitioning properties, and slowly release drug over a number of days (Adams *et al.*, 2009). Previous studies have demonstrated that the intra-articular half-life of 15-30 kDa proteins is as little as 2-4 h (Betre *et al.*, 2006a), while clinical studies have shown clearance of the 17.3 kDa IL-1Ra by 7.4 h following intra-articular delivery in the human body (Chevalier *et al.*, 2009). These short half-lives for soluble protein drugs argues for the delivery in depot forms that can sequester the drug from the soluble compartment, thus providing for sustained release

following dis-equilibria from the drug-loaded depot. Because of the rapid inflammatory response immediately after joint trauma, we wished to provide immediate and sustained exposure of intra-articular tissues to IL-1Ra to help reduce the acute inflammatory response post-fracture. This study confirmed that IL-1Ra is detectable in serum up to at least 5 d after injection, indicating that xELP depots can provide a sustained IL-1Ra release that is not obtainable with a single intra-articular injection of non-conjugated IL-1Ra. Similarly, sTNFR^{II} was also detectable in the serum of mice 5 d after intra-articular injection. The large difference in size between IL-1Ra (17.3 kDa) and sTNFR^{II} (150 kDa) indicates that xELP can encapsulate a range of different sized molecules, underscoring its potential to be used for the delivery of a variety of drugs of many sizes. There are a wide range of ELPs that have been designed for various applications, such as drug delivery and tissue engineering (Betre *et al.*, 2006a; Chilkoti *et al.*, 2002a; Chilkoti *et al.*, 2002b; MacEwan and Chilkoti, 2009; Megeed *et al.*, 2002; Nettles *et al.*, 2010); currently, ELP is being used in type-2 diabetes human clinical trials to deliver glucagon-like peptide-1, a diabetes drug (Amiram *et al.*, 2013), and has the potential to influence the treatment of other diseases in the future.

Biomarkers have been used to non-invasively identify and monitor the progression of diseases such as PTA (Hazell *et al.*, 1995; Lindhorst *et al.*, 2000; Saxne and Heinegard, 1992). Levels of certain serum and synovial fluid biomarkers, such as COMP, may be indicative of cellular processes and the level of damage caused by joint disease. Levels of COMP, HA, and IL-6 were previously examined in an acute mouse fracture model with HA significantly upregulated acutely after fracture (Lewis *et al.*, 2013). Macrophage chemokines such as MDC and TARC were selected for examination since our previous work demonstrated significant upregulation in gene expression in mouse strains after a tibial plateau fracture (Lewis *et al.*, 2013) and in cytokine levels in humans after ankle fracture (Furman *et al.*, 2014a). Human clinical studies have also shown that TGF- β_1 correlates with the degree of fracture healing in long bone fractures (Sarahrudi *et al.*, 2011; Zimmermann *et al.*, 2005; Zimmermann *et al.*, 2007), so we examined the levels of free active and total TGF- β_1 in serum. Matrix metalloproteinase 3 (MMP-3) has been associated with tissue breakdown and remodeling in arthritis; previous studies have shown some correlation with OA (Kubota *et al.*, 1997; Mahmoud *et al.*, 2005; Ribbens *et al.*, 2002), so we examined the levels of serum MMP-3 in our study. Although our biomarker analyses revealed relatively few differences between the treatment groups, we saw correlations of biomarkers with both joint tissue and bone properties, highlighting the complex interaction of bone, joint tissue, and biomarkers following trauma. Because the majority of these differences were seen at 4 weeks, the biomarkers that we measured - COMP, TGF- β_1 , IL-6, HA, MMP-3, MDC and TARC - may be better predictive of changes that occur during the acute phase of injury rather than the 4 to 8 week period after injury that we examined.

The promising results with xELP[IL-1Ra] could provide a novel adjunct therapy combined with surgical

stabilization of the joint to reduce or prevent PTA. The use of IL-1Ra has been FDA approved for RA and shown to have short-term efficacy for pain and function after soft tissue injury in the knee (Kraus *et al.*, 2012). ELP is currently being used in diabetes clinical trials (Amiram *et al.*, 2013), which would allow for the ease of transitioning xELP[IL-1Ra] from the laboratory to human trials. Nonetheless, future studies are needed to examine the long-term safety and efficacy of this approach as a therapy for PTA in humans.

Conclusions

Currently, surgical restoration is the primary treatment for articular fractures; however, PTA frequently develops following joint trauma despite appropriate surgical reduction and fixation. Our study identifies the potential for prolonged anti-cytokine therapy to serve as an adjunct therapy to current surgical stabilization. IL-1Ra decreased the osteoarthritic damage caused by PTA, indicating that IL-1 plays a role in the destruction caused by PTA after articular fractures. The use of xELP for sustained intra-articular delivery of a therapeutic in the form of xELP[IL-1Ra] holds great promise as a new treatment for PTA.

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Discussion with Reviewers

Reviewer I: Are there any signs of a humoral immune response to the large dose of ELP-matrix delivered in the joint? Is this a potential concern?

Authors: There was no evidence of a humoral immune response to the dose of ELP delivered to the joint in any of the mice sacrificed in our study. ELP was used as a drug depot because of its non-immunogenic properties; previous studies by Urry and coauthors have shown that repeated injections of ELPs into Balb/c, DBA, and CB6F1 mice failed to produce specific hybridomas, and therefore, no immune response to the material (Urry *et al.*, 1998). On the cellular level, studies by Picciolo *et al.* (1993) showed that ELPs were not recognized as a foreign substance when introduced to cells, leading to no immune response. Based on these and other studies, ELPs are not a potential concern for a humoral immune response when used as an intra-articular drug depot.

Reviewer I: It is not clear whether the ELP matrix serves a protein delivery role unique to its composition and degradative properties, or simply functions to impede diffusion of the loaded protein drug and thus provide prolonged delivery.

Authors: ELPs in general can support both of these roles; in our study, the ELP matrix served to impede diffusion of the loaded IL-1Ra or sTNFRII to provide prolonged delivery to the joint. A previous study conducted by our group examined the effect of a single intra-articular injection *versus* a systemic administration of IL-1Ra and sTNFRII separately. It was shown that systemic administration of the drugs *via* osmotic pump (IL-1Ra) or subcutaneous injections (sTNFRII) did not provide a benefit, nor did local delivery of sTNFRII (Furman *et al.*, 2014b). A single local intra-articular injection of IL-1Ra reduced cartilage degeneration, synovial inflammation, and did not affect bone morphology following articular fracture. The idea behind using the ELP was to take the dosage that was delivered in the single local injection – 0.9 mg IL-1Ra – cut it in half, and keep it in the joint space longer. One of the issues with using IL-1Ra for the treatment of OA is that it has an incredibly short half-life (4.7 h) (Chevalier *et al.*, 2009), so the fact that we could detect IL-1Ra in the joint space at 5 d demonstrates that the ELP is acting to prolong the delivery of the drug to the injury site.

Reviewer I: How long does the ELP matrix remain in the joint?

Authors: We did not directly measure ELP following intra-articular delivery. However, IL-1Ra and sTNFRII were detectable at day 5, suggesting that the ELP matrix is likely to still be in the joint 5 d after an intra-articular injection. Beyond that time, it is unknown whether the matrix still resides in the joint. Previous studies using a non-cross-linked formulation of the ELPs showed that 90 % of a 30 μL injected dose of ELP will be cleared by the joint space in 14 d (Betre *et al.*, 2006a). The cross-linked

ELP used in our study serves as a unique drug depot, and this is the first report of the intra-articular use of ELPs for drug therapy.

Reviewer I: In Fig. 2 the level of the TNF inhibitor measured in the serum is 30-100× higher than IL-1Ra, despite 3× more IL-1Ra being loaded into the ELP matrices. Is this considered a genuine difference or possibly a difference in the sensitivity of the respective ELISAs?

Authors: This measurement is considered a genuine difference, which we speculate arises from the pharmacokinetics of sTNFR_{II} and IL-1Ra. When these drugs clear the joint space after an intra-articular injection, they can be detected in the systemic circulation until they are eliminated from the body. Systemically, sTNFR_{II} has an average elimination half-life of 3-5 d, while IL-1Ra has an average elimination half-life of 3 h (Nijkamp and Parnham, 2005). Because of the short half-life of these drugs compared to other similar inhibitors, it is preferred to deliver these drugs *via* intra-articular injection instead of systemically to ensure that the drug reaches its target before it is eliminated.

Reviewer II: Could you better describe the affinity of the ELP to the IL-1Ra and sTNFR_{II}? Can you modulate this interaction so that you can deliver them more precisely temporally?

Authors: IL-1Ra and sTNFR_{II} are not bound to the ELP, but encapsulated through physical entrapment. When the drug solution is mixed with lyophilized cross-linked ELP at 4 °C and subsequently injected into the joint space at 37 °C, it undergoes an inverse thermal phase transition, causing the ELP to aggregate and form a drug depot inside the joint. This depot then slowly disaggregates, producing a prolonged release therapy. We speculate that the difference in release rate of the two drugs is due to their relative size difference; IL-1Ra has a size of 17.3 kDa, whereas sTNFR_{II} has a size of 150 kDa.

Reviewer II: Do you envision using added exogenous cells to the delivered material?

Authors: At the present time, there is no plan to add exogenous cells to the delivered material. In previous studies, we have shown that ELP can support chondrogenesis of human adipose-derived adult stem cells without exogenous chondrogenic supplements (Betre *et al.*, 2006b). In other studies by our group, we observed that intra-articular delivery of purified MSCs from C57BL/6

and MRL/MpJ mice prevented PTA after articular fracture, presumably due to an anti-inflammatory effect of the cells (Diekman *et al.*, 2013). A future application could be the delivery of both cells and drugs *via* ELPs to the knee to improve joint healing after articular fracture.

Reviewer II: How would these results relate to larger animals? Humans?

Authors: The findings of this study show the promising potential of ELPs as an intra-articular drug delivery therapy. The translation of this work to larger animals should simply require scale up of the amount of drug and ELP used to account for the larger joint space of these animals, although the clearance and degradation times may differ in larger animals. Transitioning to humans would require an FDA safety trial, as both ELPs and IL-1Ra are approved for use in human patients. Currently, ELP is being used as a subcutaneous drug depot in a diabetes clinical trial to deliver glucagon-like peptide-1 (Amiram *et al.*, 2013), which would help with transitioning the use of cross-linked ELP from bench to clinic. IL-1Ra is approved for use in RA patients, and when administered intra-articularly following injury, has shown short-term efficacy in reducing pain and improving function scores after ACL tears (Kraus *et al.*, 2012). Further clinical trials would be needed to examine the long-term safety and efficacy of xELP[IL-1Ra] as a therapy for PTA.

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