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A phase I clinical trial shows safe, sustained, AAV-mediated expression of IL-1Ra in the human osteoarthritic knee joint

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One Sentence Summary:

Intraarticular injection of sc-rAAV2.5IL-1Ra is safe, stably increasing IL-1Ra concentration in joints, providing a potential osteoarthritis therapy.

OVERLINE: GENE THERAPY

Editor's summary:

A safe gene therapy for knee OA

Osteoarthritis (OA) in the knee joint is typically accompanied by elevations in inflammatory signaling, including interleukin-1 (IL-1), in the intra-articular space. Protein and small molecule therapies delivered to the intra-articular space, including the endogenous inhibitor of IL-1 (IL-1 receptor antagonist (IL-1Ra)), are cleared rapidly from the joint and therefore new strategies to enrich therapeutics for long term treatment within the joint are needed. Here, De La Vega *et al*. take a step toward a safe gene therapy for patients with OA in a first-in-human, single center, phase I clinical trial to test the safety of intra-articular delivery of an adeno-associated virus expressing IL-1Ra.

Abstract

Osteoarthritis (OA) is a major global health problem with no disease-modifying therapies. Interleukin-1 (IL-1) is critical cytokine associated with the pathophysiology of OA and can be inhibited by IL-1 receptor antagonist (IL-1Ra). Here, we tested the delivery of a gene therapeutic encoding the human IL-1Ra to the knee in a phase 1, open-label clinical trial that enrolled nine patients with radiographic knee OA. The IL-1Ra gene was delivered by a self-complementary (sc) recombinant adeno-associated virus (rAAV) serotype 2.5 (sc-rAAV2.5IL-1Ra) by intra-articular injection into an index knee at one of three doses, low- (1×10^{11} viral genomes (vg)), mid- (1×10^{12} vg), or high- (1×10^{13} vg). The primary outcome was safety. There were no serious adverse events (AEs) related to sc-rAAV2.5IL-1Ra. Two AEs occurred that were possibly related to the vector. Both were effusions with increased pain and resolved with conservative treatment. sc-rAAV2.5IL-1Ra did not cause changes in vital signs, physical findings, or clinical laboratory measures. Less than 1% of the injected dose of sc-rAAV2.5IL-1Ra vg was detected in circulation after one day and was cleared within a week. Titers of neutralizing antibodies to AAV2.5 rose in serum and synovial fluid. In all cases, IL-1Ra concentration increased in the synovial fluid and IL-1Ra concentrations remained elevated after one year. Baseline pain and function scores improved during the study. Therefore, we found that intra-articular gene therapy with sc-rAAV2.5IL-1Ra was safe. The sustained increase in local IL-1Ra in human knee joints supports the further clinical examination of this therapy to provide therapeutic benefit in OA.

INTRODUCTION

Osteoarthritis (OA) is increasingly prevalent in aging societies and a leading source of pain, disability, and economic loss (*1*). Current treatment is often pharmacological and restricted to topical and oral analgesics with limited benefits and considerable toxicity. Intra-articular therapies for OA, most commonly corticosteroids, are cleared quickly from the joint cavity, resulting in short-term benefits (*2*). Although a recent publication indicated sustained benefit from intra-articular corticosteroids (*3*), this study lacked a placebo group, so it was not possible to determine the true duration of benefit. A Cochrane Review (*4*) suggests a short-term effect of intra-articular corticosteroids, lasting only a few weeks. There are no licensed, disease-modifying drugs that might beneficially affect one or more of the tissue pathologies in OA.

The pathogenesis of OA is complex and involves multiple tissues including cartilage, synovium, subchondral bone and, in the case of the knee, the infrapatellar fat pad (*5*). Interleukin-1 (IL-1) has properties consistent with being a critical driver of catabolic and inflammatory processes in joints with OA, which positions IL-1 as a potential therapeutic target. The endogenous inhibitor of IL-1 signaling, the interleukin-1 receptor antagonist (IL-1Ra), is therefore a possible therapeutic protein.

Rapid efflux from the joint is a particular problem when attempting to deliver anti-arthritic proteins, such as IL-1Ra, to osteoarthritic joints in a targeted and sustained fashion. Indeed, a clinical trial in which recombinant IL-1Ra (Anakinra) was injected into knees with OA produced only transient clinical improvement because of its rapid removal from the joint space (*6*). Intra-articular delivery of cDNAs encoding anti-arthritic gene products to cells within individual joints with OA offers an elegant solution to the problem of delivering proteins to joints in a sustained fashion (*7, 8*). The genetically modified cells will continue to synthesize the

therapeutic products locally for the life of the cell, resulting in persistence of transgene expression in the injected joints thus reducing, and possibly eliminating, the need for re-dosing. Local, intra-articular gene therapy of this type also reduces costs and the likelihood of adverse, off-target effects.

In preclinical testing, a variety of viral and non-viral vectors have been evaluated as agents of gene transfer to joints in an ex vivo or in vivo fashion, and their efficacy tested in animal models of arthritis (*8*). This work has led to a small number of clinical trials (*9*). In the first transfer of an exogenous gene to a human joint, retrovirally transduced autologous synoviocytes expressing IL-1Ra were injected into joints with rheumatoid arthritis (RA) (*10*). This approach, although successful, was abandoned as the advantages of adeno-associated virus (AAV) as a vector for gene delivery to joints became appreciated (*11*) and technology improved to allow efficient in vivo transduction of cells in joints (*12*).

The first human trial using AAV for intra-articular delivery injected a vector encoding etanercept, a tumor necrosis factor-alpha (TNF-α) antagonist, into joints with RA (*13*) but further development was halted after the death of a trial participant, although this was deemed unrelated to the investigational product (*14-16*). Systemic anti-TNF trials have failed to demonstrate benefits in OA. A recent Phase I clinical trial in which AAV encoding interferon-beta (IFN-β) was injected into joints with inflammatory hand arthritis was stopped because of persistent, severe tenosynovitis after only 4 of a projected 12 patients were treated (*17*). The only other published human clinical trial data concern the ex vivo delivery to joints with OA of transforming growth factor-beta 1 (TGF-β1) using retrovirally-transduced, allogeneic chondrocytes (*18*). This product received approval from the Korean regulatory authorities (*19*) which was rescinded after uncertainties emerged about the identity of the allogeneic cells. This

product is now in two Phase III trials in the US (ClinicalTrials.gov Identifiers: NCT03291470, NCT03203330). A Phase I trial using high-capacity adenovirus to deliver IL-1Ra to knee joints with OA is also underway (ClinicalTrials.gov Identifier: NCT 04119687).

We have developed a self-complementary, recombinant AAV vector serotype 2.5 encoding IL-1Ra (sc-rAAV2.5IL-1Ra) to deliver IL-1Ra to joints. Pre-clinical studies confirmed the ability of this vector to transduce both synoviocytes and chondrocytes in situ after intra-articular injection, with minimal leakage to the systemic circulation and no lasting transduction of extra-articular cells (*20-22*). Neutralizing antibodies to AAV2.5 were generated in response to intra-articular injection of the vector, but no cell-mediated immune responses were detected in these animals. Pre-clinical studies in rats and horses confirmed safety and, in horses, demonstrated sustained intra-articular IL-1Ra expression with no structural progression in joints with experimental OA (*21*). Horses also became less lame, indicating an analgesic response to the gene therapy.

Here we report data from a single center, first-in-human, Phase I clinical trial in which sc-rAAV2.5IL-1Ra was injected into one knee of 9 patients with mid-stage OA (Kellgren-Lawrence (KL) score 2-3) of the index knee (ClinicalTrials.gov Identifier: NCT02790723). The findings suggest that the treatment is safe, capable of raising intra-articular IL-1Ra concentrations in a sustained fashion that may be of potential therapeutic benefit in OA.

RESULTS

Enrollment, dosing and follow-up

Following written informed consent and screening procedures, nine patients, 6 female and 3 male, were enrolled into the study. Patient demographics (**Table 1**) and their concurrent medications were noted (**table S1**). At the initial visit, baseline assessments were performed and patients were given a single intra-articular injection of sc-rAAV2.5IL-1Ra into the index knee joint at a dose of 1×10^{11} viral genomes (vg) (low dose; patients 001-003), 1×10^{12} vg (mid-dose; patients 004-006) or 1×10^{13} vg (high dose; patients 007-009). Participants were followed for 52 weeks according to the schedule of assessments (**table S2**). Eight individuals completed the entire follow-up period. Patient 001 was unavailable for the final, 52-week visit because of COVID-19 lock-down.

Intra-articular injection of sc-rAAV2.5IL-1Ra is safe in 9 human participants

The primary outcome of this study was safety. Adverse events (AEs) reported during this study are summarized in **Table 2**. No AEs led to discontinuation or early withdrawal of the participants. There were no serious adverse events (SAEs) related to the intra-articular injection of sc-rAAV2.5IL-1Ra and no SAEs that were considered to be dose-limiting. There was one SAE that was considered unrelated to the study drug, a traumatic fracture of the right metatarsal which healed uneventfully.

Two participants, patient 004 (mid-dose, 1×10^{12} vg cohort) and patient 009 (high-dose, 1×10^{13} vg cohort), experienced an effusion in the index knee after injection of the vector. Patient 004 experienced a mild, delayed effusion at 4 weeks accompanied by an increase in Western

Ontario and McMaster Universities Osteoarthritis Index (WOMAC) pain score from 2 to 6, and an increase in Numeric Rating Scale (NRS) pain from 0.5 to 1.5. These symptoms resolved with rest and ice, and were absent by the 12-week office visit with concomitant improvement in pain. The relationship to sc-rAAV2.5IL-1Ra is unclear since this patient had a history of knee effusions prior to study entry. Patient 009 had an effusion that began within 24 hours after injection and resolved with rest and ice. This individual then went on to have another moderate effusion which began 3 days after the first one resolved, became mild within a couple of weeks, but had not fully resolved until week 12. Reflecting these events, NRS pain scores increased from a baseline value of 2 to a value of 4 at 2 weeks and then declined at subsequent time points. The WOMAC pain score decreased from a baseline value of 3 to a value of 1 at 4 weeks, rising to a value of 4 at 12 weeks before declining at subsequent time points. In both these patients, the AEs were deemed possibly related to sc-rAAV2.5IL-1Ra. Both resolved with rest and ice, and did not require steroid injection. Patient 005 reported knee pain during the 11^{th} month post-injection period that had resolved with conservative treatment by the time of the 52-week visit. This event was deemed unlikely to have been related to the study drug.

Injection of sc-rAAV2.5IL-1Ra did not alter vital signs or clinical laboratory values.

There were no changes in vital signs or physical findings related to the study treatment. Clinical laboratory measures, including blood chemistry, liver function, and complete blood count did not meaningfully change during the study (data file S2). Although systemic IL-1 inhibition may cause neutropenia (*23*), neutropenia was not observed at any time following intra-articular delivery of sc-rAAV2.5IL-1Ra.

The sc-rAAV2.5IL-1Ra genome was detected transiently in peripheral blood and in synovial fluid of some patients

Quantitative polymerase chain reaction (qPCR) was used to measure vg in peripheral blood, synovial fluid, and urine at predetermined time intervals (**table S2)**. Vector genome copies (vgc) were detected in the peripheral blood of all patients, except patient 008, 24 hours after intra-articular injection of sc-rAAV2.5IL-1Ra (**Fig. 1, A and B**). The circulating amount of vgc was highly variable between patients but never exceeded 1% of the total administered dose and declined rapidly, being near the lower limit of detection one-week after injection. There did not appear to be a direct relationship between the dose administered and the number of vgc measured in whole blood. In certain patients, very low vgc numbers were detected at later time points (**data file S1**). Vgc were not detected in the peripheral blood of patient 008 at any time point.

In synovial fluid, a large number of vgc was only observed in the injected knee of patients 004 and 009 and only in the one-month post-injection aspirate. Patient 009 also showed a very low vgc signal in synovial fluid at week 12 (**Fig. 1, C and D**). Extremely low or undetectable amounts of vgc were present in the synovial fluids of all other patients at any of the sampled time points. Vgc were not detected in the urine of any patient at any time.

Injection of sc-rAAV2.5IL-1Ra generated a humoral, but not a cell-mediated, immune response to the viral capsid

Most patients (8/9) had low pre-existing neutralizing antibody (NAb) titers (< 1:8) to AAV2.5 at baseline. NAb titers in serum rose mildly after injection of low dose vector, but increased more substantially in the sera of all participants injected with mid- and high doses of vector, with the exception of patient 008 (**Fig. 2, A and B**). Patient 008 had a moderate pre-existing titer (1: 512

in serum; 1: 1024 in synovial fluid) NAb at baseline which remained relatively stable during the course of the study. NAb titers in synovial fluid (**Fig. 2, C and D**) broadly reflected the pattern seen in sera. Enzyme-Linked Immunosorbent Spot (ELISpot) measurements assessing IFN-γ production by activated T-cells were negative for all patients at all time points through week 12, suggesting that patients did not mount a T-cell response to AAV2.5 (data file S3). Since the T-cell response is known to peak at approximately 4 days (*24*) and all samples had returned negative values, testing at subsequent time points was not performed.

Intra-articular injection of sc-rAAV2.5IL-1Ra elevated IL-1Ra concentrations in synovial fluid

Baseline concentrations of IL-1Ra in synovial fluid ranged from 107 – 283 pg/ml (average 160 ± 69 pg/ml). In each of the 8 patients from whom synovial fluid could be aspirated, concentrations of IL-1Ra increased after injection of vector (**Fig. 3**). In patients 001 and 003, who received the lowest dose of vector, synovial fluid IL-1Ra concentrations rose approximately 5-fold by 12-26 weeks (**Fig. 3A**). Synovial fluid was not available for patient 001 at the 52-week time-point because of COVID-19 lockdown. However, at 26-weeks concentrations of IL-1Ra remained elevated. Patient 002 had no retrievable synovial fluid at any time. Elevated IL-1Ra concentrations were maintained for the entire 52-week follow-up in patient 003.

Concentrations of IL-1Ra in synovial fluids aspirated from patients receiving the mid- and high doses of vector were substantially higher than those receiving low dose vector, but patient-to-patient variability obscured any potential difference between the mid- and high dose groups in this small trial (**Fig. 3, A and B**). The high expression of IL-1Ra (9,425 pg/ml) in the synovial fluid of patient 004 at 4 weeks coincided with an increased knee effusion. Likewise, patient 009 (3,668 pg/ml) in the high dose group also showed a peak of expression at a time of

increased effusion. Collectively, the data show that IL-1Ra concentrations in the synovial fluid of the injected knee were elevated one month after injection of sc-rAAV2.5IL-1Ra, with greater IL-1Ra expression in the mid- and high- dose groups compared to the low-dose group. In 5 of the 8 patients who provided synovial fluid aspirates, IL-1Ra concentrations remained elevated at or near peak amounts for the entire follow-up period. In three patients, (004, 008 and 009) synovial fluid IL-1Ra concentrations decreased from peak values at 4 weeks, but remained elevated above baseline at 52 weeks. IL-1Ra concentrations in serum at entry were 301.6 ± 95.2 pg/ml (mean ± S.D.). There was no consistent elevation in systemic IL-1Ra concentrations during the study (**table S3**). There was no apparent relationship between concentrations of IL-1Ra in synovial fluid (**Fig. 3**) and serum (**table S3**).

We also evaluated the presence of inflammatory cytokines, including IL-1, in the synovial fluid. IL-1α was undetectable in any synovial fluid sample tested. Low concentrations of IL-1β were detected sporadically in certain synovial fluid aspirates (**table S4**). There was insufficient synovial fluid for consistent measurement of other cytokines. IL-1α or IL-1β were not detected in any serum sample at any time.

Intra-articular injection of sc-rAAV2.5IL-1Ra reduced pain and improved function

Patient-reported outcomes (PROs) were collected using standardized instruments. Pain was determined both by the NRS pain scale (0-10) and the WOMAC 3.1 pain subscale score (Likert scale, 0-20). In both scales, a lower score means less pain. There was no minimum criterion for pain at entry and scores ranged from 0/10 to 8/10 at baseline (**Table 1**). Patient 008 scored zero on both NRS and WOMAC pain scales. Patients in the low dose cohort reported a marked reduction in NRS pain that was sustained for 52 weeks in patients 002 and 003, and for 26 weeks

in patient 001 (**Fig. 4A**). Patient 001 could not be evaluated at 52 weeks because of COVID-19 lockdown. The effects of vector administration on pain were harder to discern in the patients receiving the mid- and high doses of sc-rAAV2.5IL-1Ra because their baseline pain scores were relatively low (**Fig. 4, A and B and Table 3**). However, all scores declined during the course of the study.

Overall, WOMAC pain scores in the low dose cohort (**Fig. 4, C and D and Table 3**) showed an improvement of variable duration in response to injection of sc-rAAV2.5IL-1Ra. In the mid- and high- dose cohorts, participants reported improvements in pain that were more sustained (**Fig. 4, C and D**). Patient 006 entered the study with a NRS score of 7 and a WOMAC pain score of 12; these values improved over time and dropped progressively to zero NRS and 3.5 WOMAC at 26 weeks. At the end of the study, the NRS score remained zero and the WOMAC pain had further declined to a value of 1. No patients had a higher NRS or WOMAC pain score at the end of the study than on entry. WOMAC function scores and WOMAC total scores followed a similar trajectory as those reported for WOMAC pain (**Fig. 4, E to H and Table 3**).

Radiologic assessment shows little change during the course of the trial

Of the eight patients in the study who were available for 52-week follow-up, six showed no evidence of disease progression on X-ray and five showed no evidence of disease progression on MRI (**table S5**). Of the remaining patients, two showed slight deterioration on X-ray, but changes were not consistent across imaging modalities and likely within measurement error. MRI suggested that one patient (Patient 004) may have experienced some cartilage loss. There were no increases in synovitis in 7 of 8 patients, with one participant (Patient 004) showing a minor increase. Overall, given the small patient numbers, the radiological findings indicated no

274 change or minimal progression as might be expected over a 12-month period, with no evidence
275 of rapidly progressive OA.
276

DISCUSSION

This single-center, open-label, phase I study demonstrated that intra-articular injection of sc-rAAV2.5IL-1Ra into human knee joints with radiographic OA was safe up to the maximum 1×10^{13} vg dose evaluated. There were no drug-related SAEs. Two patients experienced post-injection effusions that were possibly related to injection of sc-rAAV2.5IL-1Ra, but symptoms subsided with conservative treatment. Injection of sc-rAAV2.5IL-1Ra raised synovial fluid concentrations of IL-1Ra which remained elevated during the entire 52-week follow-up. Patient reported outcomes provided evidence of sustained clinical responses, but with small group sizes and in the absence of a placebo group it is not possible to draw strong conclusions.

Previous clinical trials involving the intra-articular injection of recombinant AAV are restricted to the delivery of etancercept in joints with RA (*13, 14*) and IFN-β in finger joints with inflammatory arthritis, including both RA and OA (*17*). There was no further development of the former strategy following the death of a patient in a Phase II study, although the death was not attributed to the administered study material (*15, 16*). The second study was discontinued as a result of SAEs (*17*). Here, we report a first-in-human trial where the gene therapy has been shown to be safe, and where intra-articular expression of the transgene product has been confirmed and shown to persist. Moreover, we show preliminary evidence, albeit anecdotal, of a sustained clinical response warranting further study.

The safety data described here differ from a prior study that reported the discontinuation of a Phase I trial using AAV5 to deliver IFN−β into finger joints because of a persistent, severe tenosynovitis in response to injection of the vector *(17)*. Such a SAE was not observed in the present study, possibly because of differences in AAV serotype, transgene, manufacturing and purification process, joint, and dose. Of these, dose may be particularly important. In the prior

study 1.2×10^{12} vg AAV were administered to the carpometacarpal joint and 0.6×10^{12} vg AAV to the proximal interphalangeal joint (*17*). Given the small size of these joints compared to the knee, this represents a considerably higher dose than used in the present study. Because the tendon sheath is extra-articular, the occurrence of tenosynovitis raises the additional possibility that vector was injected inaccurately or escaped from the joint after injection. Similar to our study, Vrouwe *et al.* did not report the use of any immune-suppressive conditioning agents that are frequently used in clinical gene therapy protocols (*25*).

Two patients in our study, one of whom had a history of knee effusions, experienced effusions after injection of sc-rAAV2.5IL-1Ra. Knee effusions following intra-articular AAV have been described previously as administration site reactions (*14*). These are unlikely to have been infections, as they did not have typical clinical presentation of fever and they resolved with conservative treatment. The effusions may reflect an immune response to the viral vector. Addition of an immune conditioning regimen, as applied in many human clinical trials with AAV, may reduce the immune response and this possibility is being evaluated in a Phase Ib clinical trial (ClinicalTrials.gov Identifier: NCT05835895).

In addition to the absence of drug-related SAEs, there were no changes in vital signs or physical findings related to treatment. Likewise, clinical laboratory measures, including blood chemistry, liver function and complete blood count did not exceed normal limits during the course of the trial. Neutropenia was not observed, which was closely monitored because neutropenia is a recognized side effect of systemic IL-1 inhibition (*23*). Consistent with the lack of systemic sequelae, the concentration of circulating IL-1Ra (301.6 $\pm$ 95.2 pg/ml) did not change markedly during the study, an observation in agreement with preclinical data (*20-22*), and consistent with values reported in the literature for healthy individuals (*26*).

In synovial fluids, the baseline range of IL-1Ra concentration was 107 – 283 pg/ml. There is no consensus in the literature on the expression of IL-1Ra in joints with arthritis. In prior work, IL-1Ra was not detected in synovial fluids from joints with OA, unlike fluids from joints with RA (*27*); conversely, others have reported a range from 292–1951 pg/ml in fluids from joints with OA (*28*). We were unable to detect IL-1 in sera and most synovial fluids, which agrees with previous research showing that the concentration of IL-1 in synovial fluid from joints with OA is in the picogram range and notoriously difficult to measure (*29-31*). Intra-articular expression of the transgene was confirmed by the elevated concentrations of IL-1Ra present in the synovial fluid after injection of sc-rAAV2.5IL-1Ra. More IL-1Ra was expressed at the higher two doses of vector, but differences between the latter two doses were not discernable because of inter-patient variability in expression within the small group sizes. Elevated expression of IL-1Ra persisted for the entire 12-month follow-up, but declined from its peak values in patients 004, 008 and 009.

Patients 004 and 009 developed an effusion which may be associated with an inflammatory state leading to the subsequent decline in IL-1Ra expression. If so, it may be worth exploring the use of corticosteroids as anti-inflammatory agents to examine the relationship between elevated inflammation and the expression of the transgene in future trials. Although expression of IL-1Ra was later curtailed in the two patients with effusions, earlier samples taken from their knee joints revealed considerable elevations in IL-1Ra expression. In a previous preclinical study, where the equine version of sc-rAAV2.5IL-1Ra was injected into equine joints with experimental OA, IL-1Ra expression was elevated and correlated with the degree of inflammation (*20*). Whether this relates to our observations in humans is unclear, but one possible explanation is that the cytomegalovirus (CMV) enhancer-promoter used to drive

transgene expression may be responsive to inflammation or other disease-associated signals in joints.

The limited off-target biodistribution of vector genomes after intra-articular injection provided an additional safety measure. In agreement with pre-clinical pharmacokinetic data (*20-22*), less than 1% of the administered dose was detected in peripheral blood on the day after injection and this had been cleared by one week. No vector DNA was detected in any of the urine samples. Only one participant, patient 008, entered the study with a moderately high titer (1:512 in serum; 1:1024 in synovial fluid) of pre-existing NAb to AAV2.5. This is consistent with earlier research showing that approximately 16% of patients with OA have moderate titers of NAb against AAV2.5 (*33*). Unlike other patients in the trial, titers of NAb in patient 008 did not change greatly after dosing. The presence of NAb in this participant did not appear to prevent successful transduction of articular cells, an observation of relevance to patient selection and re-dosing in future trials. Re-dosing with sc-rAAV2.5IL-1Ra or an equivalent vector using a different AAV serotype is an important matter for future research. In vitro experiments using sera and synovial fluids from patients with high titers of NAbs will facilitate this research.

No cell-mediated immune responses to AAV2.5 were detected in any patient, but high titers of NAb developed in synovial fluid and serum of individuals receiving the mid- and high doses of sc-rAAV2.5IL-1Ra. Although it is well established that administration of AAV provokes a robust humoral immune response in experimental animals and humans, cell-mediated responses are only seen in humans, possibly as a result of a memory response to previous infections (*32*). That such responses were not seen in the present study probably reflects the relatively low dose of administered vector and its precise, ultra-sound guided, local delivery into an isolated, enclosed body cavity. This is reassuring, because cell-mediated immune responses to

AAV capsids, that were not observed in the corresponding pre-clinical studies, have emerged as efficacy issues in human clinical trials (*32*).

The patient reported outcomes provided some evidence of clinical responses, both in terms of pain and function, but the small number of patients, the absence of a placebo group, and the low baseline pain values in some patients preclude firm conclusions. At the lowest vector dose, improvements were modest and not sustained in all patients. However, at the mid- and high doses there was evidence of a more durable response which was particularly evident in patient 006 who entered the study with high pain and low function scores that, in a delayed fashion, normalized during the study period. Overall, all scores improved during the course of the study and in no patient were the NRS or WOMAC scores higher at the end of the study than on entry.

Determination of whether the improved scores were sufficient to achieve a minimal clinically impart different (MCID) is complicated by the lack of consensus on what constitutes a MCID (*34*). Moreover, five patients entered the study with low pain and WOMAC scores that confound this type of analysis. However, three patients (002, 006, 007) spontaneously volunteered satisfaction with their response to the treatment. The imaging data were also limited by small numbers but generally showed no or mild structural progression, consistent with what might be expected in a 12-month study. Further determination of the clinical importance of the concentrations of exogenous IL-1Ra reported here in knee OA will require the identification of robust biomarkers, which presently do not exist as surrogate endpoints in OA. Candidate biomarkers are being evaluated in a larger, Phase Ib clinical trial (ClinicalTrials.gov Identifier: NCT05835895) along with clinical and structural endpoints.

This study has several limitations including the small number of patients in the trial, the absence of a placebo group and the fact that some key baseline characteristics of the enrolled

patients, such as mean BMI, WOMAC and NRS scores, differed substantially (**Table 1**). The low baseline pain scores of several participants reflects the use of radiographic scores rather than pain scores as the key entry criterion in this Phase I study whose primary outcome was safety and tolerability. Patients were only followed for 52 weeks, so it is not possible to determine whether transgene expression and symptomatic improvement persisted beyond one year with disease-modifying effects. Moreover, patients were allowed to continue taking pre-existing medications thereby complicating the interpretation of any symptomatic improvement. These matters will be the focus of subsequent clinical trials. Finally, the design of the study did not allow us to identify which cells in the joint were transduced by the vector; however, prior pre-clinical equine data demonstrated transduction of chondrocytes and synoviocytes (*20, 21*). AAV2.5 has also been shown to transduce mesenchymal stem cells in mice (*35*).

Collectively, the results of this Phase I study suggest that the intra-articular injection of sc-rAAV2.5IL-1Ra into human knees with radiographic OA is safe and can elevate the intra-articular concentration of IL-1Ra in a sustained fashion with the possibility to improve pain and function. These findings justify larger, well-powered trials to confirm safety and efficacy, and determine whether there is a disease-modifying effect. Selection of patients for determination of a disease-modifying effect could be based on OA phenotypes, such as individuals with more inflammatory disease as indicated by imaging synovitis using ultrasound or MRI, serum biomarkers such as elevated serum C-reactive protein or by genotypic stratification based, for instance, on polymorphisms in the IL1RN gene (*36-38*). Such a trial could compare clinical and biomarker outcomes to placebo or an active comparator, such as an intra-articular corticosteroid.

MATERIALS AND METHODS

Study design

This was an open-label, uncontrolled, unblinded, Phase I trial designed to assess the safety and tolerability of sc-rAAV2.5IL-1Ra when introduced by intra-articular injection into knee joints of participants with definite radiographic OA (KL score of 2-3). Nine patients were enrolled into the study, a number typical for Phase I, novel, exploratory, gene therapy trials. In the absence of relevant, prior human data, a meaningful power analysis was not possible. An early endpoint would be triggered by product-related SAEs. Safety was defined as the absence of a SAE. Tolerability was defined as the absence of an AE severe enough to cause a patient to drop out of the trial. Primary and secondary outcome measures are given in **Table 4**. The protocol was approved by the FDA (IND # 16717) and the Mayo Clinic (IRB # 15-007542) and registered on ClinicalTrials.gov (Identifier: NCT02790723). Patients were screened at the Mayo Clinic and, if interested, qualified individuals were consented and offered participation in this trial according to key entry and exclusion criteria (**tables S6 and S7**). Written, informed consent was obtained after the nature and possible consequences of the studies were explained. Once consent was obtained, concurrent medications (**table S1**), baseline clinical assessments and blood samples were collected (**table S2**). After synovial fluid aspiration, enrolled baseline values were established (**table S2**), and qualifying patients received a single, intra-articular injection of sc-rAAV2.5IL-1Ra into the index knee joint; a 2 ml volume of viral suspension was injected at each dose under ultrasound guidance. JS administered the vector to patient 001; JLS administered vector to all other participants apart from patient 4 to whom vector was administered by a qualified fellow of JLS. In patients with bilateral disease, the more symptomatic knee was selected as the index joint. Prior to injection of sc-rAAV2.5IL-1Ra, synovial fluid was aspirated from the index knee under ultrasound guidance. The following day the injected knee joint was

examined for signs of inflammation and swelling. Patients underwent clinical evaluation at 1 day, 1 week, 2 weeks, 4 weeks, 12 weeks, 26 weeks and 52 weeks (final visit) after injection of sc-rAAV2.5IL-1Ra. Continuation of existing medications (**table S1**) and unrestricted activity were allowed following injection of the vector. Nine patients were enrolled into the study on a rolling basis and were divided into 3 cohorts of 3 patients who each received low- (1×10^{11} vg), mid- (1×10^{12} vg), or high- (1×10^{13} vg) dose of sc-rAAV2.5IL-1Ra. This was a dose-escalation study, so the lowest dose was administered first. There was a gap of at least 2 weeks between patients at any given dose by which time the previous patient had undergone at least 2 follow-up visits to confirm absence of SAEs. There was a gap of at least one month between cohorts. This ensured that each patient at a lower dose had undergone at least 4 follow-up visits before the dose was escalated. At the times indicated in **table S2**, patients provided a NRS score for pain (0-10), and WOMAC scores for pain (0-20), stiffness (0-8), and function (0-68) at the time of the office visit. A total WOMAC score was generated by adding the three individual WOMAC scores and normalizing to 100%. In all outcome measures, a lower score signifies lower disease activity.

Patient assessment

History, physical, vital signs, and concurrent medications were noted in the procedure room. Complete blood counts, blood chemistry, liver function tests and prothrombin time were conducted by Mayo Clinic Laboratories, a facility certified by both the College of American Pathologists and the Department of Health and Human Services through the Clinical Laboratory Improvement Amendments (CLIA). X-ray and MRI were performed in the Department of Radiology, Mayo Clinic. Patients underwent a fixed flexion, weight bearing X-ray with KL grading by an experienced radiologist at the Mayo Clinic. The KL score was determined by the

compartment with greatest degree of OA. Joint space width was measured on a Picture Archiving and Communication System (PACS) workstation using a standard caliper tool measuring distance from cortical bone of femoral condyle to cortical bone of tibial plateau.

Non-contrast enhanced MRI imaging of the index knee was performed using a General Electric 3.0T MRI scanner (GE Healthcare) with the following sequences: sagittal proton density (PD) without fat saturation, sagittal, coronal and axial T2-weighted with fat saturation, coronal T1-weighted without fat saturation, and T2 mapping sequence in all three planes. Semiquantitative readings according to the MRI Osteoarthritis Knee Score (MOAKS) were performed by a qualified radiologist, blinded to dose, at the Mayo Clinic.

Cytokine Assays

Enzyme-Linked Immunosorbent Assay (ELISA) measurement of IL-1Ra and other cytokines was performed using commercially available ELISA kits **(table S8)** following validated, standard operating procedures at the Immunochemical Core Laboratory of Mayo Clinic. The Immunochemical Core is certified by both the College of American Pathologists and the Department of Health and Human Services through CLIA. For synovial fluid, which was available in limited volumes, cytokines were assayed in the following priority sequence until the sample ran out: IL-1Ra, IL-1β, IL-1α, IL-6, IL-8, fibroblast growth factor (FGF)-2, TGF-β_1, TNF-α. (volumes used: 40 µl for TGF-β_1; 100 µl for TNF-α; 200µl for IL-1Ra, IL-1β, IL-6, IL-8, FGF-2; 400 µl for IL-1α).

Immunoassays

NAbs and vector genomes were measured in the Mayo Clinic Musculoskeletal Gene Therapy Laboratory. NAb titers were measured by their ability to inhibit the transduction of human embryonic kidney (HEK293) cells (ATCC number CRL-1573) by AAV2.5 encoding green fluorescent protein (AAV2.5.GFP) (*33*). Heat inactivated samples of serum or synovial fluid from study participants were serially diluted at a 1:2 ratio and incubated with AAV2.5.GFP for 90 min at 37°C before adding to monolayers of HEK293 cells in a 96-well plate at a multiplicity of infection of 15,000 - 20,000. After 48 hours, GFP expression was measured in a microplate reader with multi-point bottom reading capabilities at excitation and emission wavelengths of 482 nm and 502 nm, respectively (Varioskan Lux, Thermo Fisher Scientific). Samples were run in triplicate.

Blood samples were assessed for the presence of anti-AAV2.5 T-cells using a previously qualified ELISpot assay (*22*). Peripheral blood mononuclear cells were isolated from whole blood and cryopreserved samples were shipped to BioAgilytix for evaluation. Cells were thawed and tested for IFN-γ production after culturing in the presence of peptide fragments derived from the AAV2.5 amino acid sequences on an ELISpot plate containing a membrane pre-coated with anti-IFNγ capture antibody. After completion of the incubation period, cells were removed, and the captured cytokine was detected by an anti-IFNγ secondary antibody conjugated to biotin followed by Streptavidin-alkaline phosphatase (Strep-AP). A substrate to Strep-AP (CTL-TrueBlue Substrate) was then added which the Strep-AP converted to a detectible insoluble colored product. Each cell-producing IFNγ was thereby detected as a colorimetric spot on the membrane. Immune activation was reported as the number of spots per well or number of spot forming units (SFU).

Measurement of AAV genomes by qPCR

Detection of vector genomes was performed according to FDA guidelines for "Long Term Follow-Up After Administration of Human Gene Therapy Products". Genomic DNA (gDNA) was extracted from whole blood using the QIAamp DNA Blood Mini Kit (Qiagen). For synovial fluid and urine, gDNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen). A standard curve was generated for each qPCR run by serial 10-fold dilutions of the AAV plasmid in TE buffer from 2.5 x 10^9 - 25 copies/µl. One microliter of standard plus 7 µl water was used for each reaction. Standard curves were deemed acceptable if the R^2 value was > 0.98. Sample values were deemed acceptable if within the 99% confidence interval provided by the qPCR software Agilent Aria v1.71 (Agilent Technologies). Primers corresponding to the SV40 sequence spanning into the IL-1Ra sequence within the AAV plasmid were designed using the PrimerQuest Tool (Integrated DNA Technologies); forward:

5' CCTCAGTGGATGTTGCCTTTA, reverse: 5' GAACAGCAGCAGGGTAATCA. Individual 20 µl PCR reactions were performed in 96-well plates using the PowerUp SYBR Green Master Mix (Applied Biosystems), plasmid DNA, nuclease-free water, and 300 nM of each primer in an Agilent AriaMx Real-time PCR system (Agilent Technologies) over 40 cycles. The assay was first developed and validated for control human gDNA from primary synovial fibroblasts (female; genetically unmodified) and HEK293 cells. Duplicate wells were used for the standard curve, control gDNA, and control gDNA plus the full range of the standard curve. The provided forward and reverse primer sequences were validated as being sensitive and specific for the AAV plasmid and used for all further assays. The limit of quantitation (LOQ) for each PCR run was determined to be 25 copies per reaction, which was the lowest number of copies that could be reliably measured. Each reaction used 500 ng of gDNA from blood or 1 µg of gDNA from urine.

Because the yield of gDNA from synovial fluid was low, 300 ng of gDNA were used per reaction. Following FDA guidelines, samples were tested in triplicate wells with the third sample spiked, and the standard curve was performed in duplicate. Samples of gDNA from each body fluid were tested for the presence of AAV genomes. A sample was determined as negative if the Cq values were <LOQ and positive when ≥ LOQ. In cases where the Cq value for the spiked well was <LOQ, the sample was considered as non-informative and repeated once.

Vector manufacture

The vector, sc-rAAV2.5IL-1Ra, is a self-complementary, recombinant AAV, serotype 2.5, containing the full coding sequence of human IL-1Ra. The coding sequence has been codon-optimized for greater expression of IL-1Ra under the transcriptional control of the CMV enhancer-promoter. Good Manufacturing Practice-Source plasmids were produced by Aldevron and used to manufacture sc-rAAV2.5IL-1Ra using GMP by the Research Institute at Northwestern Children's Hospital (RINCH; now Andelyn Biosciences). The vector was generated using a triple transfection process in adherent HEK293 cells. The harvested vector was purified using ultracentrifugation followed by ion-exchange chromatography. The final product formulation consisted of sc-rAAV2.5IL-1Ra in a sterile solution of in TMN200P buffer (20 mM Tris pH 8.0, 1 mM $MgCl_2$, 200 mM NaCl) plus 0.001% poloxamer 188. The drug product was characterized and released using qualified assays at RINCH or at their qualified vendors.

AAV titer was determined by qPCR-based DNase Resistant Particle (DRP) assay using primers targeting the CMV promoter. The infectious unit titer was based on the $TCID_{50}$ format of AAV dilutions on RC32 cells. Vector DNA identity was determined by purification of encapsidated viral DNA followed by library generation and sequencing by Ilumina MiSeq 2x250 bp Next Gen sequencing. Vector capsid protein identity was performed by western blotting using

antibodies specific to AAV VP1, 2 and 3. The full to empty ratio was determined by analytical ultra-centrifugation. HEK293 host cell protein was determined by ELISA. Replication competent AAV was assayed by qPCR after transduction and serial passage of HEK293 using AAV REP2 target.

Statistical analysis

Because of the small number of participants in each group, descriptive statistics were used for data analysis. Data are presented as the mean of technical replicates for each sample. All individual-level tabular data is available in **data file S1**.

List of Supplementary Materials

Tables S1 to S8

MDAR reproducibility checklist

Data file S1

Data file S2

Data file S3

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Author contributions: AK, TWC, PDR and SCG developed the vector used in this trial. TLS, JBB, and ZWP accrued patients to the trial and served as study coordinators. JLS, JS administered the study agent and performed synovial fluid aspiration. SJW performed synovial fluid aspirations. MLJ served as medical monitor. CJM prepped patients for treatment and follow-up, and ensured appropriate transfer of biopsy fluids. MAF performed radiological assessment of patients. RDLV, MJC, GPH, and TYA performed laboratory assays. CHE conceived the study, acquired funding and wrote the first draft of the manuscript. PC, JLS, MAF, RDLV, CHE, AK, TWC, PDR and SCG performed data analysis. All authors edited the manuscript.

Competing interests: CHE is a co-founder of Genascence Inc. and a consultant for L&J Bioscience, Cellastra Inc. and Orthogen AG. PDR is a co-founder of Genascence Inc and a consultant for L&J Bioscience, Innate Biologics, Glo-Pharma, Infinity Research Labs and Itasca Therapeutics. AK is a co-founder of Genascence Corp. TWC is a co-founder of Genascence and has held board or consulting positions with Ethris GmbH, Cardiac Risk Inhibitors Inc., Exhaura Ltd., and Catena Biosciences. PGC has done speakers bureaus or consultancies for AbbVie, Alfasigma, Eli Lilly, Eupraxia, Formation Bio, Genascence, GSK, Grunenthal, Janssen, Kolon TissueGene, Levicept, Medipost, Moebius, Novartis, Sandoz, Stryker & Takeda. JLS has consulted for AutonomUS. SCG is a co-founder of Genascence Inc. JS, REDLV, TYA, TLS, MJC, ZWP, CJM, GPH, SJW, MLJ, MAF declare that they have no competing interests. Patents related to this work include “Methods and compositions for treating osteoarthritis” WO2020112853A1 (TWC, CHE), “Methods and compositions for treating osteoarthritis” WO2024206333A2 (TWC, AK), “IL-1Ra cDNAs” WO2018106956A2 (SCG). All other authors declare they have no conflicts of interest.

Data and materials availability: All data associated with this study are in the paper or supplementary materials, data file S1 and Dryad dataset titled, "15 007542 Clinical Study Report vF (01-Feb-2023) RAW DATA" (doi:10.5061/dryad.4qrfj6qn9). Non-proprietary materials are available on request to CHE. Further information is available on the Genascence website under the ”Our Science” tab (https://genascence.com/science/).

Figure Legends

Figure 1. Vector genome copies measured in peripheral blood and synovial fluid with time after injection of sc-rAAV2.5IL-1Ra.

(**A and B**) Quantification of vgc in blood by qPCR for individual patients in the low-, mid-, or high- sc-rAAV2.5IL-1Ra dose groups (A) or averaged by group (B) over 52 weeks. $n = 9$ patients. (**C and D**) Quantification of vgc in synovial fluid of individual patients (C) or averaged by group (D). $n = 8$ patients. Patient 001 was unavailable for follow-up at 52 weeks. Patient 002 was only able to provide sufficient blood for analysis at baseline, 1 week, and 12 weeks. Not all patients were able to provide sufficient synovial fluid for testing at all time points. Patient 002 provided no synovial fluid at any time. VGC, vector genome copies; gDNA, genomic DNA

Figure 2. Titers of neutralizing antibodies to AAV2.5 in sera and synovial fluids with time after injection of sc-rAAV2.5IL-1Ra.

(A to D) sc-rAAV2.5IL-1Ra was injected on day 0 and titers of anti-AAV2.5 neutralizing antibody measured in serum (**A and B)** and synovial fluid (C and D) at the indicated times**.** $n = 9$ patients; Patient 002 was only able to provide sufficient blood for NAb analysis at baseline, 1 week and 12 weeks; Patient 001 was unavailable for follow-up at 52 weeks; Not all patients were able to provide sufficient synovial fluid for testing at all time points; Patient 002 provided no synovial fluid at any time. Nab, Neutralizing antibody.

Figure 3. IL-1Ra concentrations in aspirated synovial fluid with time after injection of sc-rAAV2.5IL-1Ra.

(A and B) sc-rAAV2.5IL-1Ra was injected on day 0 and the concentration of IL-1Ra in synovial fluid measured by ELISA at the indicated times. Data are given per individual within each dose

group (A) or as the mean values per dose cohort (B). $n = 8$; Patient 002 had no retrievable synovial fluid at any time; Patient 001 was unavailable for 52 wk follow-up; Patient 005 had no retrievable synovial fluid at baseline; Patient 008 had no retrievable synovial fluid at week 52; Patient 009 had no retrievable synovial fluid at baseline.

Figure 4. Patient-reported outcomes with time after injection of sc-rAAV2.5IL-1Ra. (**A and B**) NRS pain scores reported per individual within each dose group (A) or as the mean score per dose cohort (B). **(C and D)** WOMAC pain scores reported per individual within each dose group (C) or as the mean score per dose cohort (D). **(E and F)** WOMAC function scores reported per individual within each dose group (E) or as the means score per dose cohort (F). **(G and H)** WOMAC total scores reported per individual within each dose group (G) or as the mean score per dose cohort (H).

Table 1. Demographics and baseline characteristics of patients treated with sc-rAAV2.5IL-1Ra. BMI = Body Mass Index; KL = Kellgren-Lawrence score (0-4)

Patient Number	Dose	Sex (M/F)	Baseline BMI	Baseline Age (years)	Baseline KL Score	Bilateral (Bi) or Unilateral (Uni)
001	1×10^{11} vg Low Dose	F	27.0	52	3	Bi
002		F	36.2	50	2	Bi
003		M	35.1	48	3	Bi
004	1×10^{12} vg Mid- Dose	F	33.9	45	2	Bi
005		M	24.8	59	2	Bi
006		F	29.1	59	3	Uni
007	1×10^{13} vg High Dose	M	27.7	49	2	Uni
008		F	21.7	66	3	Bi
009		F	22.4	39	2	Bi

Table 2. Adverse events noted during the study.

AE = Adverse Event; SAE = Serious Adverse Event

*Patient 001 was unavailable for follow-up at 52 weeks

Adverse Event	Anticipated	Severity	Relationship to Intervention	Relationship to Drug	SAE	Outcome
Patient 001 Traumatic fracture, foot	No	Severe	Unrelated	Unrelated	Yes	Resolved
Patient 001 Stiffness, swelling, triggering of hand	No	Mild	Unlikely	Unlikely	No	Unknown*
Patient 002 Dyspepsia	No	Mild	Unrelated	Unlikely	No	Resolved
Patient 002 Hematoma, arm	Yes	Mild	Definite	Unrelated	No	Resolved
Patient 002 Puncture wound by barbed wire, leg	No	Mild	Unrelated	Unrelated	No	Resolved
Patient 004 Knee effusion	Yes	Mild	Unrelated	Possible	No	Resolved
Patient 005 Knee pain	No	Mild	Unrelated	Unlikely	No	Resolved
Patient 009 Knee effusion	Yes	Mild	Unrelated	Possible	No	Resolved
Patient 009 Knee pain-effusion	Yes	Mild	Unrelated	Possible	No	Resolved

Table 3. Baseline and 52-week PRO characteristics of patients treated with sc-rAAV2.5IL-1Ra.

PRO = Patient reported outcomes; NRS = Numeric Rating Scale (0-10); WOMAC = Western Ontario and McMaster Universities Osteoarthritis Index; P = Pain (0-20); F = Function (0-68); S = Stiffness (0-8); T = Total (normalized to 100%)

Patient Number	**Baseline PRO Scores**					**52-week PRO Scores**				
	WOMAC				**NRS**	**WOMAC**				**NRS**
	P	**F**	**S**	**T**		**P**	**F**	**S**	**T**	
001	10	49	6	61	6	Patient unavailable				
002	6	31	4	39	6.5	3	19	2	23	2
003	14	46	4	63	8	10	35	4	47	1
004	2	6	0	8	1	1	4	0	5	0.5
005	6	16	0	23	1.5	3	6	0	9	0.5
006	12	36	5	52	7	1	6	0	7	0
007	4	10	2	15	2.5	0	0	0	0	0
008	0	3	2	3	0	0	2	1	2	0
009	3	13	2	17	2	0	4	0	4	1.5

Table 4. Primary and secondary outcome measures

Secondary outcome measures are listed in order of importance

CBC = Complete Blood Count; NAb = Neutralizing Antibody; NRS = Numeric Rating Scale

WOMAC = Western Ontario MacMaster University Osteoarthritis Index; MRI = Magnetic Resonance Imaging.

Outcome Measures	
Primary	Safety and Tolerability
Secondary	Effect of vector on vital signs, CBC, blood chemistry, liver function. Determination of possible escape of vector into peripheral blood and urine Determination intra-articular expression of IL-1Ra (concentration of IL-1Ra in synovial fluid) Measurement of immune response to the vector (NAb, Cell-mediated) Assessment of a possible effect on disease activity, measured by NRS, WOMAC, X-ray, MRI