Supplementary Data

MATERIALS AND METHODS

Study animals

All animal procedures were conducted in accordance with both the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the University of Florida Institutional Animal Care and Use Committee. Animals used in the study were either donated to the University of Florida or purchased from local farms and training facilities. Unless otherwise stated, the horses were housed in groups in large open paddocks, with full freedom of movement.

Generation of AAV vectors

The cDNA sequence for the native *eqIL-1Ra* cDNA[S1,S2] was codon optimized[S3] and synthesized (GeneArt; Life Technologies, Grand Island, NY) with a consensus Kozak sequence[S4] immediately upstream of the translation initiation codon and flanking recognition sequences for NotI and SacII. The engineered DNA construct was directionally inserted into the corresponding sites of the pHpa-trsk scAAV vector plasmid. In this construct, expression of the transgene is driven by the CMV immediate early promoter/enhancer.[S5] The scAAV vector construct was packaged in the AAV2.5 capsid[S6, S7] at the University of Florida Vector Core or the University of North Carolina Chapel Hill Vector Core by co-transfection of 293 cells using methods previously described.[S8] Prior to experimental use *in vivo*, scAAV.eqIL-1Ra viral vector preparations were functionally characterized *in vitro* and validated for titer and transgene expression.

Study design

Twenty Thoroughbred horses between 2 and 9 years of age and of mixed sex were used in the efficacy study. The animals were healthy and free of lameness or radiographic signs of carpal joint disease. Prior to induction of the disease model, the horses were conditioned by treadmill exercise 5 days a week for 3 weeks. For each exercise day, the horses were trotted (4–5 m/s) for 2 min, galloped ($\sim$9 m/s) for 2 min, and again trotted for 2 min. Prior to further use, the animals were randomly divided into equal treated and control groups. The animal handlers and evaluators, however, were blinded to treatment group assignment.

Following treadmill conditioning, under general anesthesia, an arthroscopic examination was performed bilaterally in both midcarpal joints. During the procedure, in one randomly assigned joint, an 8 mm osteochondral fragment was created medially off the radiocarpal bone using an osteotome aligned perpendicular to the articular surface.[S9] The fragment was allowed to remain attached to the capsular tissues. To mimic a natural injury,[S10] debridement of the parent bone[S9] was not performed. The contralateral joint in each horse was examined arthroscopically in parallel, and served as a sham-operated internal control. All horses were housed in a stall for 7 days postoperatively and received appropriate veterinary care.

Two weeks post surgery, following surgical scrub of the forelimb joints, the OCF joint of the horses assigned to the treated group received an injection of 5×10^{11} vg of scAAV.eqIL-1Ra suspended in Lactated Ringer's solution in a total volume of 5 mL. Horses in the control group received 5 mL of Lactated Ringer's solution without virus. One week after injection, the horses were returned to the 5 day a week treadmill exercise program above for 10 weeks. During training, the horses received weekly clinical examinations and lameness assessments. At the conclusion of the 10-week training period, a final arthroscopic examination was performed on both midcarpal joints. The fragment was removed, and the lesion in the parent bone was debrided and repaired. Following recovery, the animals were returned to the research herd. Digital images were collected during both arthroscopic procedures. Radiographic imaging and MRI were performed immediately prior to both arthroscopic procedures and prior to treatment. Peripheral blood, urine, and synovial fluid from both midcarpal joints were collected immediately prior to treatment and on alternate weeks thereafter for the remainder of the protocol.

One horse originally assigned to the treated group was euthanized midway through the experimental protocol due to pneumonia arising from complications during anesthesia recovery. This animal was subsequently replaced to fulfill the subject number needed for statistical analyses. All data presented are from the 10 treated and 10 control animals that completed the experimental protocol; no animals were excluded from the data

analyses. Discussion of outlying data points is clearly delineated in the text and figures.

ELISA for equine IL-1β, IL-1Ra, and PGE_2

Equine IL-1Ra (R&D Systems, Minneapolis, MN), PGE_2 (R&D Systems), and equine IL-1β (GenWay, San Diego, CA) content in biological fluids were assayed using a specific ELISA. Synovial fluids were diluted 1:1 with phosphate-buffered saline containing hyaluronidase at 50 IU/mL and incubated at 37°C for 30 min prior to analysis. For serum and synovial fluid, twofold serial dilutions in reagent diluent (R&D Systems) were generated over a wide range to account for assay variability. Each dilution series was generated in duplicate, and each diluted sample was assayed in triplicate wells. Means were calculated from samples with readouts within the boundaries of the standard curves of the respective assays.

Lameness evaluation

Subjective visual lameness assessments were performed by two qualified evaluators, appropriately blinded, with the horses on the treadmill at a walk and a trot ($\sim$4 m/s) according to guidelines of the American Association of Equine Practitioners.[S11]

For objective gait assessment, an inertial sensor-based motion analysis system was used (Lameness Locator®; Equinosis, St. Louis, MO) designed specifically to detect and evaluate lameness in horses.[S12,S13] For each weekly session, at least three measurements were taken at a $\sim$4 m/s trot on a treadmill. Each measurement was calculated from a minimum of 30 uninterrupted strides. Lameness was calculated as a vector sum using the mean maximum head difference (HDmax) and mean minimum head difference (HDmin) between the left and right strides for every stride in each measurement.[S13,S14] For each session, the means of the HDmax and HDmin from at least three measurements were used to calculate the vector sum (VS) as follows:[S12,S13]

$$VS = \sqrt{HDmax^2 + HDmin^2}.$$

For both subjective and objective assessments, mean lameness values at week 1 were used as baselines and assigned a value of 1; subsequent measurements were calculated as the percent change relative to baseline.

MRI and evaluation

MRI examinations of both carpi were performed using a Toshiba Titan (Tokyo, Japan) 1.5 Tesla high-field unit. Under general anesthesia, the horses were placed in left lateral recumbency with each carpus in partial flexion (15–25°) in a quadrature transmit/receive knee coil (QD Knee). The MRI coil and sequences were selected and optimized to be clinically applicable in live horses,[S14] and included sagittal and axial proton density (PD), dorsal T2-weighted, axial T2 short-tau inversion recovery (STIR), sagittal proton density with fat suppression (PD-FS), and sagittal spoiled gradient echo with fat suppression (SPGR-FS). Total acquisition time was approximately 1 hour 20 min for all sequences on both limbs. The MRI scans for each horse were examined by three evaluators blinded to treatment group assignment. Following review of the scans from the six MRI sequences for each midcarpal joint and time point, scores were assigned for the predominant pathologies associated with the model, including synovial effusion, synovial proliferation, severity of the osteochondral lesion, damage to articular cartilage, marrow edema in the radiocarpal bone, sclerosis of the radial carpal and third carpal bones, joint capsule edema, and capsular fibrosis, using a scale from 0 to 10, where 0 represented normal and 10 represented severe pathology.[S15,S16] Scoring was based on involvement within the midcarpal joint only. Final scores for each pathology represent means of the three evaluators. Total MRI pathology scores were determined from the sum of the individual pathologies.[S15,S16]

Arthroscopic evaluation

Both midcarpal joints of the horses in the treated and control groups were examined and imaged arthroscopically following generation of the osteochondral lesion and again at the endpoint of the experimental protocol. Video recordings and digital images collected post fracture at week –2 and prior to repair of the lesion at endpoint were scored by three evaluators (blinded to the identity of the animals and their treatment) for the size of the lesion and degree of fragment repair, integration of border zone of the defect with surrounding cartilage, appearance of surface cartilage overall, and appearance of synovium and ligaments. Based on criteria from Dymock *et al*.[S17] a scoring system from 0 to 10 was used where 0 represented normal, and 10 represented severe pathology.

Histology

The osteochondral fragment and synovial tissue removed during the endpoint arthroscopy were fixed in paraformaldehyde, decalcified, and paraffin embedded. Serial sections (5 μm) were mounted

on charged slides, deparaffinized and rehydrated, and blocked in 3% peroxide/methanol for 10 min at room temperature. Alternate sections in regions of interest were stained with hematoxylin and eosin (H&E) and toluidine blue, respectively. The section series was analyzed and graded by two blinded evaluators using a grading system adapted from McIlwraith *et al*.[S18] Briefly, articular cartilage integrity was scored based on signs of chondrocyte necrosis, cluster formation, fibrillation, and/or focal cell loss. The synovial membrane was evaluated and graded based on signs of vascularity, intimal hyperplasia, subintimal edema, and/or subintimal fibrosis. Leukocytic infiltration was scored on the same scale as a readout of inflammation. Finally, the subchondral bone and repair interface was evaluated for matrix quality, osteochondral lesions, bone remodeling, and osteochondral splitting. The total score was calculated from the sum of the individual scores.

Measurement of capsid-targeted NAb

Methods were adapted from those described by Li *et al*.[S19] Synovial fluid was digested with hyaluronidase as described for ELISA, and blood serum was incubated at 56°C for 30 min for complement inactivation. Using serumless media, a series of twofold serial dilutions were generated from the pretreated serum or synovial fluid. The diluted samples were mixed with $\sim 1 \times 10^9$ vg scAAV.eqIL-1Ra packaged in AAV2.5 capsid in a total volume of 250 μL and incubated for 1 h at 37°C to allow antibody binding. The mixtures were then added to confluent cultures of primary equine synovial fibroblasts in 24-well plates containing 250 μL of serumless culture medium (500 μL/well total volume). After incubation for 48 h under standard culture conditions, the conditioned media were harvested and assayed for eqIL-1Ra content by ELISA. NAb titers were indicated as the inverse of highest dilution capable of reducing by 50% the eqIL-1Ra levels produced by cells infected with AAV.eqIL-1Ra pre-incubated as above, but without biological fluids.

Statistical analysis

Analyses consisted of independent sample *t*-tests, analysis of covariance *t*-tests with baseline scores serving as covariates, and correlational analyses. In most cases, one-tailed tests were employed, since, a priori, it was hypothesized that the horses receiving treatment with scAAV.eqIL-1Ra would have lower mean values from the diagnostic assessments employed, thus dictating the direction of the tail. The experimental layout was a two-sample repeated-measures design, with horses randomly assigned to one of the groups (treated or control). The data were analyzed using multiple independent sample *t*-tests. With a type I error of 0.05, 80% power, and an effect size of $d = 1.3$ (large), a total of 20 horses in the study were required to show a treatment effect at any time. As it was anticipated that correlations would exist between baseline measurements and the repeated measurements, the power for the study was >80% with the inclusion of baseline measurements as covariates.

SUPPLEMENTARY REFERENCES

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