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ARTICLE

Safety and biodistribution assessment of sc-rAAV2.5IL-1Ra administered via intra-articular injection in a mono-iodoacetate-induced osteoarthritis rat model

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Interleukin-1 (IL-1) plays an important role in the pathophysiology of osteoarthritis (OA), and gene transfer of IL-1 receptor antagonist (IL-1Ra) holds promise for OA treatment. A preclinical safety and biodistribution study evaluated a self-complementary adeno-associated viral vector carrying rat IL-1Ra transgene (sc-rAAV2.5rIL-1Ra) at 5×10^8, 5×10^9, or 5×10^{10} vg/knee, or human IL-1Ra transgene (sc-rAAV2.5hIL-1Ra) at 5×10^{10} vg/knee, in Wistar rats with mono-iodoacetate (MIA)–induced OA at days 7, 26, 91, 180, and 364 following intra-articular injection. The MIA-induced OA lesions were consistent with the published data on this model. The vector genomes persisted in the injected knees for up to a year with only limited vector leakage to systemic circulation and uptake in tissues outside the knee. Low levels of IL-1Ra expression and mitigation of OA lesions were observed in the vector-injected knees, albeit inconsistently. Neutralizing antibodies against the vector capsid developed in a dose-dependent manner, but only the human vector induced a small splenic T-cell immune response to the vector capsid. No local or systemic toxicity attributable to vector administration was identified in the rats as indicated by clinical signs, body weight, feed consumption, clinical pathology, and gross and microscopic pathology through day 364. Taken together, the gene therapy vector demonstrated a favorable safety profile.

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INTRODUCTION

Osteoarthritis (OA) is the most common form of arthritis and a leading cause of disability among the elderly population worldwide. In the United States, an estimated 27 million adults had OA in 2005 (ref. 1). The prevalence is projected to increase because of OA's association with aging and obesity.[2] Typical OA symptoms include pain, swelling, and stiffness in joints commonly in hands, knees, hips, or spine. OA patients may suffer a reduced quality of life or become disabled as the disease progresses. Current treatments for OA are symptomatic or palliative, and there is no cure for OA. Patients with severe OA often require joint replacement surgery in order to maintain mobility.[3]

Compelling evidence suggests that interleukin-1 (IL-1), a key inflammatory cytokine secreted by chondrocytes and cells within the synovium, is an important intra-articular mediator of cartilage loss and inflammation during OA development.[4] IL-1 increases the levels of matrix-degrading enzymes and reduces the synthesis of extracellular matrix proteins by chondrocytes.[2] Moreover, IL-1 mediates hyperalgesia and pain in the OA joints by acting on the nociceptive system.[5] The IL-1 receptor antagonist (IL-1Ra), a physiologic inhibitor of IL-1 signaling, holds promise as a biologic treatment for OA. Indeed, intra-articular injections of recombinant human IL-1Ra

are able to protect against the development of experimentally induced OA lesions in dogs.[6] However, a single, intra-articular injection of IL-1Ra was not effective in treating OA of the human knee,[7] probably because of the rapid clearance of IL-1Ra from the joint.[8]

Because OA is a chronic disease, a therapeutic agent such as IL-1Ra needs to be present in diseased joints for extended periods of time. Intra-articular gene transfer of IL-1Ra cDNA is a promising approach to delivering the IL-1Ra protein to the joint for OA treatment. Several proof-of-concept studies have achieved promising results in this regard. For example, delivery of equine IL-1Ra cDNA via adenoviral vector into joints of horses with experimentally induced OA leads to elevated intra-articular expression of IL-1Ra protein for 4 weeks and also significantly reduces the severity of OA.[9] Furthermore, studies in which synoviocytes or chondrocytes are transduced *in vitro* by lentiviral or retroviral vectors containing the IL-1Ra cDNA and are then transplanted into animal joints with OA also demonstrate significant chondroprotective effects.[10–12] *Ex vivo* delivery of IL-1Ra cDNA to human rheumatoid joints has also been achieved.[13,14]

Using recombinant adeno-associated viral (rAAV) vector to transfer IL-1Ra cDNA into joints is more expeditious for clinical application because of its safety profile, *in vivo* delivery, and long-term expression

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potential. Gene transfer using self-complementary rAAV (sc-rAAVIL-1Ra) produces a therapeutic IL-1Ra level in inflamed rabbit knees.[15] Likewise, AAV-mediated human IL-1Ra (sc-rAAV-hIL-1Ra) transgene delivery into horse forelimb joints effectively transduces synovial fibroblasts and articular chondrocytes and provides biologically relevant hIL-1Ra expression.[16] *In vitro* transfer using selected serotypes of sc-rAAV vector also showed success in both equine and human synovial cells.[16] Collectively, these preclinical studies consistently demonstrate the efficacy of intra-articular IL-1Ra cDNA gene transfer for OA treatment.

We performed an investigational new drug–enabling pre-clinical safety study to evaluate toxicity and biodistribution of sc-rAAV2.5-IL-1Ra in a rat model of OA, to support approval of first-in-human trials for the vector in patients with OA. Because the human IL-1Ra protein may potentially cause an immune response in rats, the study tested three dose levels of the rat IL-1Ra cDNA vector and one high dose level of the human IL-1Ra cDNA vector that would be used in the clinical trial. General toxicity endpoints, including clinical signs, clinical pathology, gross and microscopic pathology, as well as immunogenicity and biodistribution, were evaluated throughout a year after intra-articular vector administration.

RESULTS

The study design and group designations are summarized in Table 1, and the details are described in the Materials and Methods section. The rats, including control animals (group 1), were administered mono-iodoacetate (MIA) by intra-articular injection to induce OA lesions and 3 days later were intra-articularly administered vehicle or vector. The day of vector (or vehicle) injection was designated as study day (SD) 1 for each animal.

Evaluation of general toxicity (mortality, clinical signs, body and organ weights, and clinical, macroscopic, and microscopic pathology)

Five rats from various dose groups (two from group 1, on SD 98 and 175; one from group 2, on SD 287; and two from group 4, on SD 79 and SD 271) underwent moribund euthanasia and one rat from

group 2 was found dead on SD 326. On the basis of macroscopic and microscopic examination, none of the moribund euthanasias or premature deaths was attributable to the vector dosing.

No vector dose-related adverse clinical signs were observed in the rats. Transient signs of abnormal gait, limping, and shaking were observed in a few animals, which as expected were consequences of the cartilage injury induced by the MIA administration.

Among male and female animals, group mean body weights were not statistically different among the vehicle dose group and the vector dose groups at any time point. Body weights increased over time during the study, and the growth curves of the vector-treated males or females were comparable with their corresponding vehicle controls (Figure 1).

Feed consumption data were quite variable. Sporadically, mean daily feed consumption was statistically significantly different between the vehicle control group and a vector dose group. However, there were no clear trends of vector dose-dependent differences in males or females (data not shown).

Hematology and clinical chemistry parameters (see Supplementary Table S1 for a complete list of parameters) were analyzed for the main study animals euthanized at SD 7, 26, 91, 180, and 364. There were no alterations in hematology or clinical chemistry parameters that were attributed to treatment with the sc-rAAV2.5rIL-1Ra or sc-rAAV2.5hIL-1Ra vector. While statistically significant differences between vehicle control and vector dosed groups were identified for a few hematology and clinical chemistry parameters, the changes were slight or were without a dose response, only identified in one sex, or not consistently identified over time (Supplementary Table S2).

Weights of major organs were measured at necropsy for all main study animals, and relative organ-to-body weights and organ-to-brain weights were also calculated. Absolute and/or relative weights of several organs in the vector dose groups were significantly different from those in their corresponding control groups only at SDs 7, 91, and 180 (*i.e.*, the differences were not observed at SDs 26 and 364). In general, the statistically significant organ weight changes lacked a dose–response relationship, and/or there was no

Table 1 Study design

Group	Treatment	300 µg MIA (right knee)[a]	Vector dose[b] (vg/right knee); no left knee injection	Number of animals per group (M/F) at euthanasia time[c]				
				SD 7	SD 26[d]	SD 91	SD 180	SD 364[e]
1	Vehicle control	Yes	0 (vector vehicle)	8/8	8/8	8/8	8/8	8/8
2	Low-dose rat vector	Yes	5×10^8	8/8	8/8	8/8	8/8	8/8
3	Mid-dose rat vector	Yes	5×10^9	8/8	8/8	8/8	8/8	8/8
4	High-dose rat vector	Yes	5×10^{10}	8/8	8/8	8/8	8/8	8/8
5	High-dose human vector	Yes	Human, 5×10^{10}	—	8/8	—	—	—
6	Untreated	No	No	5/5[f]	—	—	—	—

MIA, mono-iodoacetate.
[a]Group 1–5 rats received an injection of MIA in the right knee and of saline in the left knee. [b]The day of vector (or vehicle) dosing was designated as study day (SD) 1. Rat vector is the vector carrying rat IL-1Ra transgene (*i.e.*, sc-rAAV2.5rIL-1Ra) and human vector is the vector carrying human IL-1Ra transgene (*i.e.*, sc-rAAV2.5hIL-1Ra). [c]For group 1–5, five of the eight males and eight females per subgroup were designated as the main study animals. The remaining three of each sex (designated as satellite animals) were included for assessment of vector concentration and transgene expression in the knee joint. [d]Males were euthanized at SDs 25 and 26 and females at SDs 26 and 27. This euthanasia time point is referred to as SD 26 throughout the text. [e]Satellite male animals were euthanized at SD 363, and this euthanasia time point is referred to as SD 364 throughout the text. [f]Group 6 animals received no treatment and euthanasia day was arbitrarily designated as SD 8 for males and SD 9 for females.

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consistency between absolute and relative weights or between males and females and thus were considered to be incidental and not attributable to the vector administration (Supplementary Table S3).

Macroscopic examination identified sporadic findings in various tissues from the main study rats in various treatment groups (Supplementary Table S4). None of the findings were considered to have resulted from vector administration. Minimal to mild foci of yellow discoloration of the soft tissues in the right knees and red discoloration of popliteal lymph nodes were identified in a few animals in both the control and vector-dosed groups, which were considered to be a consequence of the MIA-induced lesions.

The primary microscopic finding in the MIA-dosed right knees (femorotibial articulation and patella) of both the control and vector-dosed rats was chondrocyte necrosis, along with matrix proteoglycan depletion and synovial mononuclear infiltration, which progressed over time to include cartilage fibrillation/fragmentation, cartilage loss/collapse, subchondral fibrosis, bone formation/resorption, osteophyte formation, subchondral osteosclerosis, and chondrocyte hyperplasia (Figures 2 and 3 and Supplementary Table S5). These lesions suggest that OA was successfully induced by the MIA administration. The lesions are consistent with published data on this model.[17,18] Histopathological examination did not identify any adverse effects in the right knees attributable to the vector administration. There was also no obvious histopathological

evidence that the rat or human vector treatment resulted in mitigation of the MIA-induced OA lesions in the right knees.

Minimal-to-mild immune cell infiltrates were identified in the left knees (saline-injected), the skin and subcutis around the region of the injection site (both the left and right knees), and/or the draining popliteal lymph nodes in some rats at early euthanasia time points, SD 7 or SD 26. These findings were usually observed both in the control and vector-dosed animals and considered to be associated with injection procedures and/or MIA administration.

Microscopic examination of remaining tissues did not identify any lesions associated with MIA or vector administration.

Evaluation of cartilage damage in the knees by Mankin scoring
The knees from group 1 (control) and group 4 (high-dose rat vector) main study animals euthanized at SD 7, 180, and 364 were evaluated by Mankin scoring to quantify MIA-induced cartilaginous lesions in the knees. The Mankin score is frequently used for histopathological classification of OA severity, focusing on evaluation of cartilage damage, with a scale ranging from 0 (normal cartilage) to 14 (worst possible score).[19] The Mankin score results confirmed that injection of MIA successfully induced cartilage degeneration in the right knees of the rats. The Mankin scores increased with time. Moreover, the median Mankin score (when combined for males and females) of the right knees in the high-dose vector group (group 4) was significantly lower than that in the control group (group 1) at the SD 7 and SD 180 euthanasia time points, suggesting the vector treatment reduced the severity of cartilage damage. However, the possible therapeutic effect of the vector was not observed in the rats euthanized on SD 364 (Table 2).

Vector pharmacokinetics and biodistribution
Concentrations of vector genome in the blood were below the limit of quantitation (50 vg/μg genomic DNA) at 4 hours or 24 hours post-vector injection in all the control and low-dose vector animals, as well as in mid-dose vector males and some females. Some females in the mid-dose vector group (group 3) showed low levels of vector in the blood at both time points (group geometric mean of 2 vg copies/μg DNA at 4 hours and 13 vg copies/μg DNA at 24 hours). Following high-dose vector injection, both male and female animals in group 4 had moderate levels of vector genomes in the blood. Vector concentrations were higher at 24 hours (group geometric mean of 2,122 vg copies/μg DNA for males and 6,581 vg copies/μg DNA for females) than at 4 hours postinjection (group geometric mean of 43 vg copies/μg DNA for males and 74 vg copies/μg DNA for females). The females appeared to have slightly more vector leakage into circulation than the males in the same dose group (mid or high dose group) at both time points.

Vector genomes persisted in the right knees up to SD 364 following intra-articular injection in all of the vector dose groups (Figure 4). The amounts of vector in the right knees were, for the most part, dose dependent. The vector concentration in the right knees was usually highest at the SD 7 or SD 26 time point and then declined in most subgroups over time. No quantifiable vector genomes were identified in the left knees of the vector-treated animals or in either knee of the control animals.

Little systemic distribution of vector occurred following the intra-articular injection. No quantifiable vector genomes were detected in blood from SD 7 onwards. Consistent with the vector pharmacokinetics results at 4 and 24 hours, no quantifiable amounts of vector were identified throughout the study in any tissues outside the knee in rats in the low-dose vector group and males in the
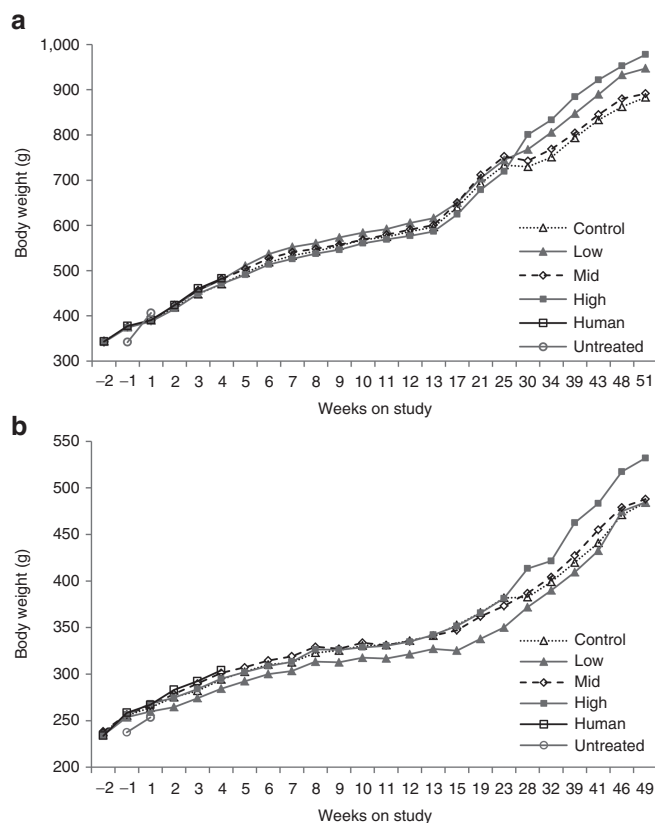

Figure 1 Body weight changes in rats administered vector or vehicle. (**a**) Males. (**b**) Females. The data are presented as the group means (data were combined from all euthanasia time point subgroups). Control: vehicle control group (group 1); low: low-dose rat vector group (group 2); Mid: mid-dose rat vector group (group 3); High: high-dose rat vector group (group 4); Human: high-dose human vector group (group 5); untreated: group 6.
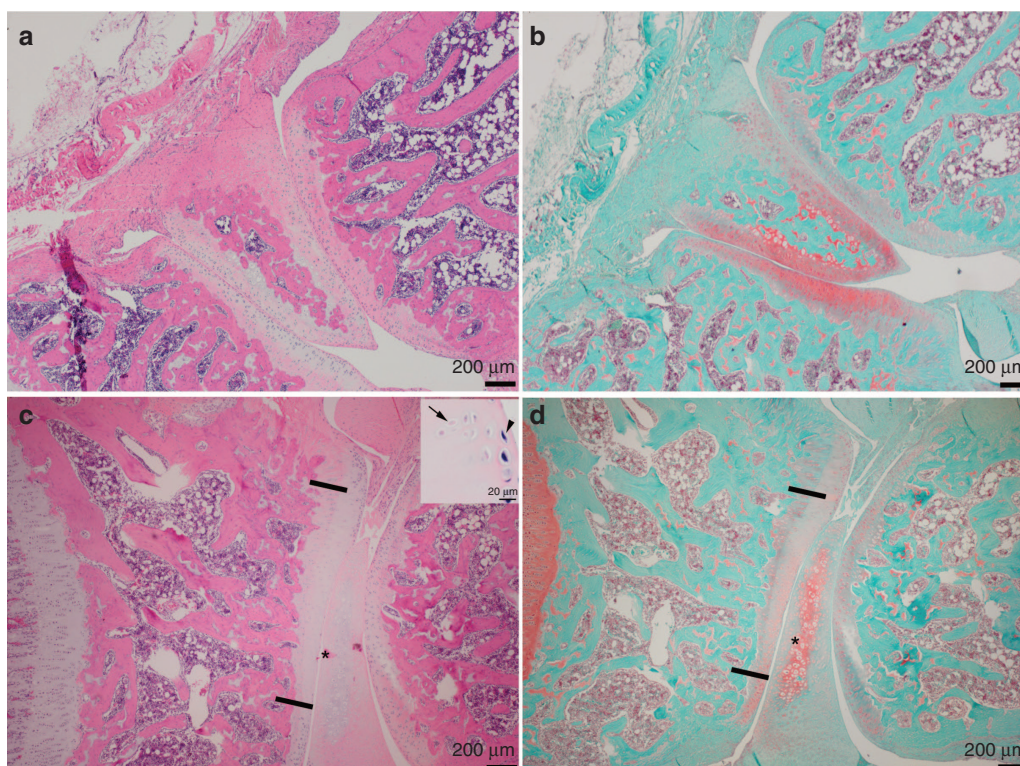
Figure 2 Chondrocyte necrosis with depletion of proteoglycans 10 days after MIA injection (SD 7). (**a** and **b**) Control knee from a control rat; (**c** and **d**) MIA-injected knee from a control rat. (**a** and **c**) Hematoxylin and eosin stain; (**b** and **d**) Safranin O Fast Green stain. In panels **c** and **d**, the cartilage of the medial tibial plateau (between the two lines) and meniscus (asterisk) contain necrotic chondrocytes. In panel **d**, reduced cartilage staining by Safranin O (red) demonstrates decreased proteoglycan content. The inset in panel **c** is a higher magnification image showing necrotic chondrocytes (arrows) and viable chondrocytes (arrowhead). MIA, mono-iodoacetate.

mid-dose vector group. For the mid- (females) and high-dose vector groups, low amounts of vector were observed only in liver, adrenal gland, spleen, bone marrow, or popliteal lymph nodes at SD 7 or SD 26. By SD 91 and after, vector was not consistently found outside the knee in any dose group. Vector distribution pattern in tissues was similar between the high-dose rat vector group and the high-dose human vector group at SD 26.

Rat and human IL-1Ra protein expression in knees and serum
In the right (MIA-injected) knees of the control animals (group 1), rat IL-1Ra protein levels appeared to be lower than those present in the corresponding left knees at SDs 7 and 26 in the females and to a lower extent at SD 7 in the males. However, there was no apparent difference in the rIL-1Ra levels between the right and left knees in the control group rats after SD 26. The loss of endogenous IL-1Ra protein in the right knees could be attributable to the MIA-induced necrosis of chondrocytes (as identified by the histopathological evaluation), one of the cells in the joint that produces IL-1Ra.

A trend toward an increase in rIL-1Ra level in the right knee was only observed in some female rats at SDs 26, 91, and 364 in the high-dose vector groups. However, the low number of samples per dose group precluded meaningful statistical analyses. Serum rIL-1Ra levels were also measured in rats treated with or without the vector at SDs 7, 26, and 364. The majority of the samples were below the lower limit of quantification (LLOQ) of the assay (391 pg/ml), and there was no clear increase in serum rIL-1Ra levels in rats in any vector dose group compared with the corresponding control group (data not shown).

Interestingly, human IL-1Ra protein expression was observed in the right knees of group 5 rats at SD 26, and the expression was

more robust in females than males. The hIL-1Ra level in the right knees was significantly higher than the corresponding left knees in the female rats, indicating that the vector injection resulted in transgene expression in the joint (Figure 5a). Consistent with the results in the right knees, the serum hIL-1Ra concentrations in the vector-dosed female animals at SD 26 were significantly higher than those in the corresponding control females (Figure 5b).

Cellular and humoral immune responses to the vector
The T-cell immune response to vector AAV2.5 capsid protein VP1 peptide pools (pp) 1, 2, and 3 was assessed using an interferon γ (IFN-γ) ELISpot assay in splenocytes from rats euthanized at SD 26 and SD 180. Overall, all of the responses were very low (below 40 spots/250,000 splenocytes) and highly variable. The statistical analysis showed that only splenocytes from group 5 (high-dose human vector group) male rats at SD 26 had significantly higher responses to pp2 than control rats (Supplementary Figure S1). However, this response was extremely low and can likely be considered biologically negligible because there were no associated findings identified in the spleens by histopathological examination.

Serum neutralizing anti-AAV2.5 antibody titers increased in a vector dose-dependent manner in both male and female animals (Figure 6). The antibody titers in mid- and high-dose vector groups rose at SD 26 and peaked at SD 91 and then declined slightly at the later time points, SD 180 and SD 364. Rats in the high-dose rat vector group and in the high-dose human vector group exhibited similar anti-capsid antibody titers at SD 26. Animals administered the low dose of vector had generally low antibody titers compared with the mid- and high-dose vector groups. In general, no apparent
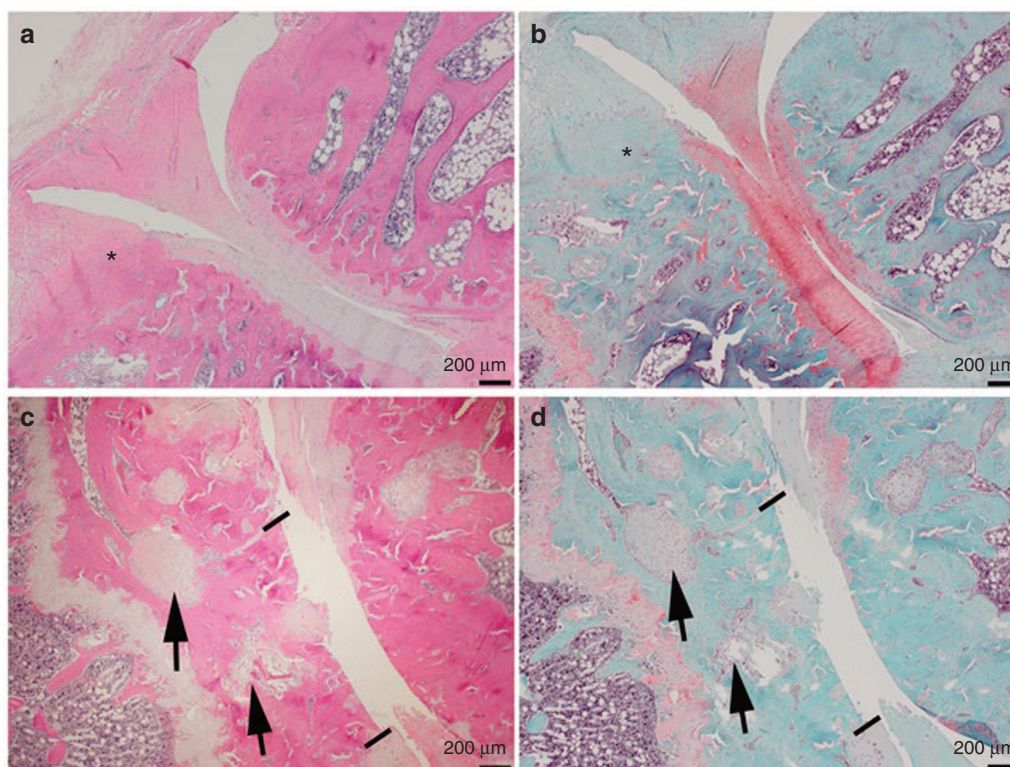
Figure 3 Cartilage loss with sclerosis of underlying subchondral bone 1 year after MIA injection (SD 364). (**a** and **b**) Control knee from a control rat; (**c** and **d**) MIA-injected knee from a control rat. (**a** and **c**) Hematoxylin and eosin stain; (**b** and **d**) Safranin O Fast Green stain. In the MIA-injected knee, the articular cartilage of the medial tibial plateau (between the two lines) and the medial femoral condyle is missing. The meniscus is fragmented, and the central portion of the meniscus is missing. The subchondral bone of the medial tibial plateau and femoral condyle is increased in thickness and contains islands of cartilage and fibrosis (arrows). In the control knee **a** and **b**, the asterisk marks an osteophyte that formed due to naturally occurring osteoarthritis. MIA, mono-iodoacetate.

Table 2 Mankin scores on knees of rats treated with vehicle or high-dose rat vector[a]

	Treatment	Sex	SD 7	SD 180	SD 364
Left knee (group 1)	Saline (MIA vehicle)	M	2.8 (2.5–2.8)	2.2 (2.2–2.3)	4.5 (3.9–5.3)
	No vector	F	3.8 (2.8–4.3)	4.0 (3.5–4.3)	6.0 (3.5–7.3)
		C	2.8 (2.6–3.6)	3.5 (2.1–4.0)	5.0 (3.8–6.3)
Left knee (group 4)	Saline (MIA vehicle)	M	2.3 (1.5–2.3)	3.8 (3.8–3.8)	5.3 (5.0–6.3)
	No vector	F	2.0 (1.8–2.0)	4.3 (3.5–5.5)	6.0 (4.8–7.0)
		C	2.0 (1.6–2.3)	3.8 (3.6–5.2)	5.6 (4.8–6.8)
Right knee (group 1)	MIA	M	4.0 (3.0–4.3)	10.0 (9.5–14.0)[b]	13.0 (11.4–13.3)
	Vector vehicle	F	4.8 (4.0–4.8)[b]	10.8 (10.5–14.0)	12.5 (11.8–12.5)[b]
		C	4.1 (3.3–4.7)	10.6 (9.6–14.0)[b]	12.5 (11.8–13.0)[b]
Right knee (group 4)	MIA	M	2.0 (1.5–4.3)	5.3 (4.8–5.3)	14.0 (11.3–14.0)
	High-dose rat vector	F	2.5 (2.5–2.8)[c]	6.0 (6.0–9.3)	12.3 (11.8–12.5)
		C	2.5 (2.0–3.5)[c]	5.6 (4.9–8.4)[c]	12.4 (11.6–14.0)

MIA, mono-iodoacetate.
[a]Data are presented as median and interquartile range (*i.e.*, first quartile–third quartile); $n = 4$–5 for M (males) or F (females) and $n = 9$–10 for C (combined). Note knees from SDs 26 and 91 euthanasia points were not evaluated. [b]The median score is significantly different from that of left knee (group 1) for the same gender. [c]The median score is significantly different from that of right knee (group 1) for the same gender.

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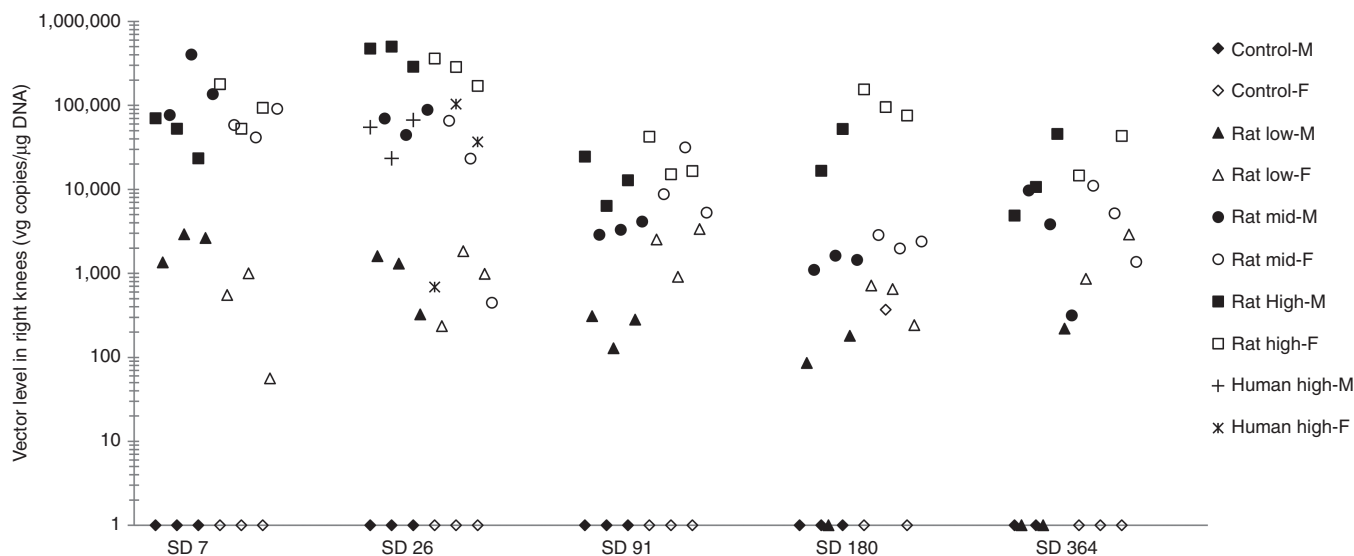G Wang et al.

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Figure 4 Vector concentrations in right knees as a function of administered dose and time. Each symbol represents vector concentration value for each animal in the indicated dose group at the indicated time point. $n = 3$ ($n = 2$ for high males at SD 180 and control males, low females, and high females at SD 364). Note that values below the quantifiable level (the LLOQ was 50 vg/µg DNA) were designated as 0 vg/µg DNA (shown as 1 µg DNA in the log scale graph). LLOQ, lower limit of quantification.
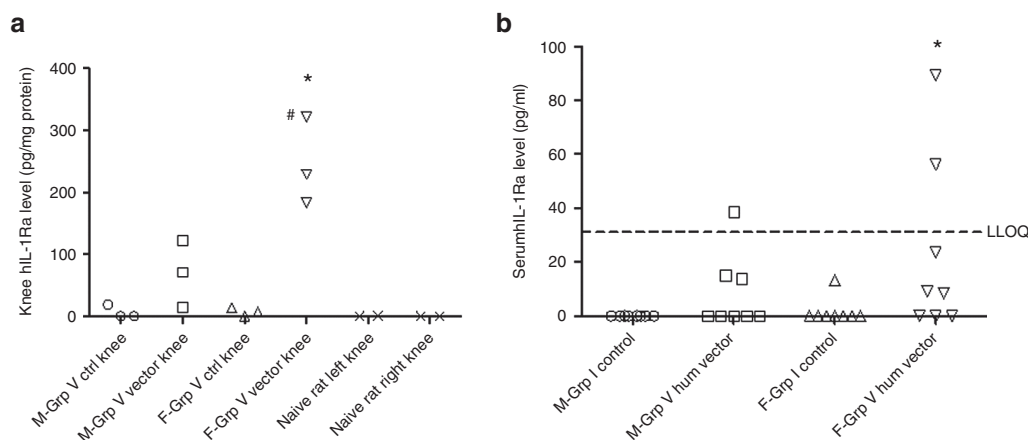
Figure 5 Concentrations of human IL-1Ra protein in (**a**) knee-capsule extracts (**b**) and serum at 26 days after administration of vector containing hIL-1Ra transgene. In panel **a**, # indicates a sample off-scale high and arbitrarily set to the highest standard concentration (2,000 pg/ml) before correction for total protein concentration; samples below the LLOQ before correction for protein are shown as X. * indicates significantly different from the left knee of group 5 females ($P < 0.05$) using Kruskal–Wallis test with Dunn's *post hoc* analysis. Human IL-1Ra levels obtained from two naive rats are also included as baselines. In panel **b**, most values were below the LLOQ of 31.3 pg/ml, indicated as a dashed line. * indicates $P < 0.05$ versus the female control animals using the Kruskal–Wallis test with Dunn's *post hoc* analysis. LLOQ, lower limit of quantification.

difference in anti-AAV2.5 antibody generation between males and females was noted in most dose groups.

DISCUSSION

The primary objectives of the study were to assess the safety and biodistribution of an AAV-mediated gene therapy vector carrying rat or human IL-1Ra transgene in an MIA-induced model of OA in rats. Intra-articular injection of MIA produced knee lesions consistent with previously reported changes in the MIA-induced OA model in the rat.[17,18] Specifically, after intra-articular MIA injection, the rat knees exhibited extensive chondrocyte necrosis and subsequent development and progression of OA. The Mankin scoring evaluation, which focuses on articular cartilage, also confirmed that injection of MIA successfully induced cartilage damage in the right knees of the rats. This suggests that the OA rat model was

established as intended to assess the safety and biodistribution of the IL-1Ra gene therapy.

Administration of the sc-rAAV2.5rIL-1Ra vector (carrying the rat IL-1Ra cDNA) by intra-articular injection at the three tested doses (5×10^8, 5×10^9, and 5×10^{10} vg/knee) caused no apparent adverse effects locally or systemically in the rats as evaluated by clinical observations, body weight, feed consumption, clinical pathology, and macroscopic and microscopic pathology through SD 364 post-vector dosing. The sc-rAAV2.5hIL-1Ra vector carrying the human IL-1Ra cDNA (the product to be used in the clinic) at the high dose of vector (5×10^{10} vg/knee) also did not exhibit vector-associated adverse effects through SD 26 post-vector dosing.

Following the intra-articular injection of the vector, there was only limited vector leakage into circulation in the high-dose vector animals and minimal leakage in the mid-dose vector females at 4
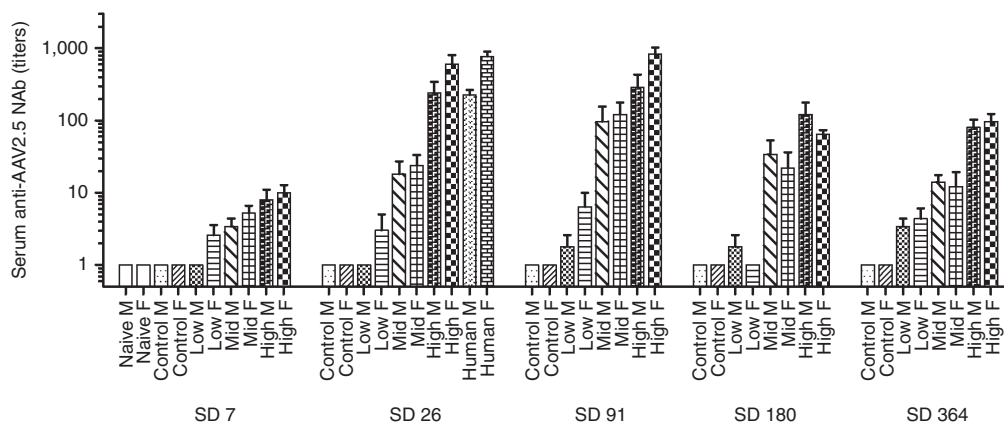
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Figure 6 Anti-AAV2.5 neutralizing antibody titers in rat serum post-vector administration. The results are presented as mean ± SEM of five animals per group. Values reported as "<5" were assigned as "0" (shown as 1 in the log scale graph). Naive: group 6; control: group 1; low: group 2; mid: group 3; high: group 4; human: group 5. F, female rats; M, male rats.

and 24 hours. By SD 7, no quantifiable vector was found in the blood of rats in any dose groups. Consistent with the limited leakage identified in the vector pharmacokinetics analysis, only low levels of vector genomes were detected outside the knee in several tissues of some high-dose vector animals and a few mid-dose vector females on SD 7 and SD 26; no quantifiable vector genomes were identified in any tissues outside the knee in the mid-dose vector male animals and low-dose vector animals on SD 7 and SD 26, or in any dose groups by SD 91 and after. The vector pharmacokinetics and biodistribution data suggest that there is no potential safety concern about extended vector persistence in off-target organs beyond the last time point evaluated in this study.

Following intra-articular injection, the vector genome persisted up to SD 364 in the right knees in a generally dose-dependent manner. In most vector dose groups, the vector concentration in the right knee peaked on SD 7 or SD 26 and then declined at the later time points. However, the vector concentrations in the knees varied among time points, particularly in the low-dose vector females. Although vector genomes were consistently present in the right knees at all the time points, increased rIL-1Ra protein expression was not consistently observed in those knees compared to the level in the right knees of corresponding control animals. Loss of chondrocytes in the right knees after MIA dosing could at least partially an explanation for low transgene expression on the basis that there might be insufficient host cells for vector transduction. Indeed, previous studies showed that AAV vector transduced both the chondrocytes and synovial fibroblasts.[15,16,20] The lack of consistent, significant rIL-1Ra expression in the right knees may be further confounded by individual animal variation, high background rIL-1Ra expression in the knees, small sample size (only three animals per group), and potential heterogeneity (knee joints along with surrounding tissues) of the knee tissues collected for analysis. However, following the human vector dose (group 5), increased hIL-1Ra expression in both the right knee and the serum of some male and female animals was observed compared to the corresponding left knees and the control animals, respectively, with the increase being statistically significant for the female rats. This suggests that the transgene expression does occur in the knee joint. The reason that the female rats exhibited better expression of hIL-1Ra than male rats is unclear. The vector genome concentrations in the knee joint were comparable between male and female rats on SD26. Gender-dependent transgene expression was previously reported in several AAV-based gene therapies, although, in those

cases, males showed greater efficacy.[21–23] In this study, the intra-articular injection is considered as a local route of administration, and male and females rats received the same vector dose and volume. It is possible that female rats have smaller knee joint volume than male rats and thus their knee cartilage and synovial tissues were subjected to greater exposure to vector solution following the injection. Indeed, there are significant gender differences in cartilage volume and surface area of men and women.[24]

Significant effects of the vector dosing on the progression of MIA-induced OA in the rats was not obvious from the histopathological examination on the overall knee joints. However, the Mankin scoring, which focuses on the evaluation of the articular cartilage of the femoral condyles, indicated that the high-dose vector treatment reduced the severity of cartilage damage in the right knees of the rats on SD 7 and SD 180. This possible therapeutic effect of the high-dose vector treatment was not observed in the rats euthanized on SD 364. The lack of effect at the longest time point is likely due to the aggressive OA model used in the study. At SD 364, OA had reached a maximum Mankin score in nearly all rats. The gene therapy slowed the rate of cartilage loss, but did not reduce it to zero; by 1 year, the treated knee joints had caught up with the untreated, OA knees.

The discrepant findings in efficacious effects between the histopathological examination and the Mankin scoring evaluation may be explained by the latter's focus on the cartilage of the femoral condyle, while the former examined the overall knee joint, and it included an assessment not only of changes in articular cartilage but also changes in the subchondral bone (resorption, new bone formation, fibrosis, bone cyst development, and sclerosis), osteophyte formation (the development of bone spurs at the joint periphery to help stabilize the joint), and synovial inflammation and proliferation. Also, the Mankin scoring was performed consistently for the same section level (at least when both condyles were present in the section at that level), whereas the histopathological assessment was performed at the level that had the most severe lesions (the most severe lesions were usually on the tibial plateaus per the Pathologist). Moreover, Mankin scoring only evaluated the slides stained with safranin O/fast green (SOFG) for evaluation of collagen and proteoglycan, but the histopathology evaluated both the hematoxylin and eosin (H&E)- and SOFG-stained slides.

The vector at similar dose range produces therapeutic IL-1Ra levels in the knee joint of horses and rabbits.[15,16] However, the obvious efficacy of the vector was not observed in the current animal model,

which may be attributed to the fast progression of the MIA-induced OA model. Proteoglycan synthesis inhibition and IL-1β expression are maximal in rats at day 2 post-MIA injection, and extensive chondrocyte degeneration occurs at day 1 post-intra-articular MIA injection; by days 5 and 7 post-MIA injection, chondrocyte necrosis, collapse of cartilaginous matrix, loss of cartilage, and subchondral bone remodeling have already occurred.[17,18,25] Therefore, at the time the vector was administered (i.e., 3 days after the MIA injection), it is likely that the lesions were already too severe to be reversed by the expression of IL-1Ra. In addition, due to extensive chondrocyte death, the vector lost the cellular basis for efficient transduction and consequent transgene expression within cartilage. However, these circumstances may not be an issue in naturally occurring OA in humans, where cartilage degeneration is slowly progressive, typically taking place over the course of months to years.

Anti-AAV2.5 NAb developed in a dose-dependent manner. The antibody titers in serum were relatively high at SD 26 and SD 91 and then declined at later time points. The reduction in vector genomes present in the tissues between SD 26 and SD 91 might be a consequence of the increased Nab titers by SD 26. Evaluation of the cellular immune response to the AAV2.5 capsid revealed no biologically significant immunogenicity following the intra-articular rat or human vector administration, in terms of IFN-γ-producing splenocytes in response to *ex vivo* stimulation by the AAV2.5 capsid peptide pools.

In conclusion, the sc-rAAV2.5IL-1Ra vector delivered by intra-articular administration showed a favorable safety profile in the MIA-induced OA rats when they were followed up to 364 days. The vector genome persisted in the right knees up to a year post-injection, but outside the knees, vector genomes were below the quantifiable level by day 91 post-dosing. The data from this study, along with previous efficacy studies, warrant further clinical development of the AAV-based IL-1Ra gene transfer into knees for OA treatment.

MATERIALS AND METHODS
Animals
Wistar rats (Crl:WI) were purchased from Charles River Laboratories (Wilmington, MA). All animal study procedures were approved by the Lovelace Respiratory Research Institute (LRRI) Institutional Animal Care and Use Committee. The animals were quarantined and housed according to Standard Operating Procedures at LRRI consistent with the Office of Laboratory Animal Welfare Guide for the Care and Use of Laboratory Animals. The animals were 11 to 13 weeks old at the time of the initial dosing. After the animals were randomized and assigned to the study groups, they were identified by using unique alphanumeric numbers encoded in a transponding microchip (Trovan, Santa Barbara, CA) placed subcutaneously between the scapulae.

Vector, MIA, and vehicle controls
Rat vector sc-rAAV2.5rIL-1Ra and human vector sc-rAAV2.5hIL-1Ra were produced using the triple plasmid transfection method in HEK293 cells, purified, and characterized at the University of North Carolina (UNC) Vector Core. The vector is a self-complementary, recombinant AAV, serotype 2.5 containing the coding region of human or rat IL-1Ra under the transcriptional control of the human cytomegalovirus (CMV) immediate early promoter. Characterization testing for the vector article included sterility, endotoxin, identity, purity, infectious titer, and viral particle titer. The vector formulation buffer (phosphate buffer with 2.7 mmol/l potassium chloride and 350 mmol/l sodium chloride, pH 7.26), also provided by the UNC Vector Core, was used as the vehicle control and diluent for the vector.

The MIA was purchased from Sigma-Aldrich (product#57858, St Louis, MO). Sterile saline (0.9% sodium chloride, for injection) obtained from Hospira (NDC#0409-4888-10, Grand Island, NE) and Nova-Tech (Cat# 510224, Rocky Mount, NC) was used as the MIA diluent and vehicle control.

Study design
The MIA-induced OA model was employed in this study for two reasons. First, spontaneous OA is extremely uncommon in rats.[26] Intra-articular injection of MIA generates an experimental OA rat model easily, quickly, reliably,

and synchronously. Second, the MIA model, unlike the majority of induced OA animal models,[26] does not involve surgery to the knee joint and thus maintains the capsule intact to mimic human OA knee capsules.

The study design is summarized in Table 1. The in-life and general toxicity endpoints of the study were conducted in compliance with good laboratory practice regulations. Following the release of the healthy rats from quarantine, the animals were randomized into dosing groups by sex and body weight, using Provantis Software (Instem, Staffordshire, UK), with 40 M/40 F in each group of groups 1–4, 8 M/8 F in group 5, and 5 M/5 F in group 6. Animals in groups 1–4 were further divided into subgroups (8 M/8 F per subgroup) by scheduled euthanasia time points. In each subgroup, 5 M/5 F rats were designated as the main study animals and the other 3 M/3 F as satellite animals to provide knee and lymph node tissues for IL-1Ra and vector biodistribution analyses. Each animal in dose groups 1–5 were administered MIA (300 µg/knee) in their right knee and saline in their left knee. The MIA dose was selected based on data obtained in a pilot study that was designed to confirm the MIA model of OA in the Wistar rats. Three days after MIA dosing (SD 4), vector (groups 2–5) or vehicle (group 1) was administered to the right knees at the vector doses specified for each dose group in Table 1. The three vector doses tested in this study, in terms of the volume of the knee joint, are equivalent to the medium, high, and 10× high proposed clinical doses. These were based on the doses previously found effective in rabbits and horses.[15,16] The day an animal received the vector (or vehicle) was designated SD 1 for that animal. The injection volume was 50 µl per knee for both the MIA and vector dosing.

Subgroups of group 1–4 rats were euthanized at SDs 7, 26, 91, 180, and 364, and the group 5 animals were euthanized at SD 26. In addition, group 6 animals receiving no treatment (naive) were euthanized at SD 8 (males) and SD 9 (females) time point and served as negative controls for the IL-1Ra measurements.

Following vector dosing, detailed clinical observations and body weight measurements were performed weekly for 13 weeks and thereafter monthly for all surviving animals. Feed consumption was collected weekly for 4 weeks and then monthly. In addition, blood was collected at 4–5 hours and 24 ± 1 hours post-vector dosing from the SD 180 euthanasia subgroups for determination of vector concentration.

The main study rats were euthanized at the scheduled time points by i.p. injection of an overdose of a pentobarbital-based euthanasia solution, Euthasol. Blood was collected by cardiac puncture into EDTA tubes for vector biodistribution and hematology analyses (Supplementary Table S1). Additional blood was collected and processed to serum for serum chemistry tests (Supplementary Table S1), as well as IL-1Ra level and antibodies to the AAV2.5 vector capsid. After a complete gross examination, the organs were weighed, and tissues were collected for analysis of vector biodistribution, IFN-γ EliSpot (splenocytes on SD 26 and SD 180), and microscopic histopathology as assigned in Supplementary Table S6. In addition, the knees and popliteal lymph nodes were collected from the satellite study animals for analysis of vector genome content, and the knee tissues and sera were also collected for IL-1Ra protein assay.

Microscopic histopathology
All fixed tissues (~40 tissues per animal, Supplementary Table S6) from the control (group 1), high-dose rat vector (group 4), and high-dose human vector (group 5) animals were evaluated. Gross lesions from the group 2 and 3 rats were also processed for histological examination. Because the high-dose vector treatment (group 4) did not demonstrate any efficacy or toxicity, only the knee joints and draining (popliteal) lymph nodes from the group 2 and 3 animals at SD 7, 91, and 364 were assessed by microscopic histopathology.

All fixed tissues, except for the knees and patellas, were trimmed, paraffin-embedded, sectioned at ~5 µm, and stained with H&E using standard histological procedures. The knees and patellas were decalcified in EDTA Versenate Decal Solution (Cat# DCVERGAL, American MasterTech, Lodi, CA). The patellas were trimmed along their longitudinal axis and sectioned to permit evaluation of the articular surface and subchondral bone. The knees were trimmed into two approximately equal halves through a frontal plane, and the caudal half was embedded with the cut plane facing down. The resulting microscopic sections generally included medial and lateral femoral condyles, tibial plateaus, and menisci. Four sections of knees were cut from each paraffin block at each of two levels, separated by ~180–220 µm. At each level, one section was stained with H&E, and a second section was stained with SOFG to evaluate the collagen and proteoglycans. The entire femorotibial joint was evaluated microscopically from sections at each level, and the section with the most severe findings was graded. The H&E and SOFG slides were evaluated by a board-certified Pathologist. The

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histopathology evaluation was semi-quantitative, and each gradable finding was assigned into a severity score of 1–4.

Mankin scoring on femoral articular cartilages

Knee joint slides (one level) stained with SOFG from the control (group 1) and high-dose rat vector (group 4) main study animals euthanized at SD 7, 180, and 364 were evaluated using Mankin Score System as previously described.[19] The Mankin scale ranges from 0 (normal cartilage) to 14 (worst possible score). Both femoral condyles on each knee were scored separately to determine the final score by two independent reviewers in a blinded manner. The average score of both reviewers was used for data analysis.

Quantitation of vector genomes in blood and tissues

Samples of blood, brain, kidneys, heart, gonads (ovaries or testes), adrenal glands, lungs, spleen, bone marrow (femur), and liver were collected at necropsy from each main study animal at all time points for vector biodistribution analysis. The vector quantity was first analyzed in all tissues (and blood) listed above for groups 1 and 4 at SD 7. Tissues from a subgroup were not analyzed if no quantifiable vector amount was detected in the same type of tissue from the higher vector dose or earlier euthanasia time point. In addition, the knee joints and popliteal (draining) lymph nodes collected from the satellite group animals at all euthanasia time points were assayed for vector concentration.

Blood and tissue samples were aliquoted and stored at −80 ± 10 °C until the time of DNA extraction. Genomic DNA extraction from blood or tissues was performed using the DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD) according to manufacturer's instructions. The sample DNA concentration was evaluated by measuring the absorbance at 260 nm (A260) and the DNA quality by a ratio of A260/A280 using a BioTek μQuant plate reader.

The vector genome copy numbers were assayed by qPCR using a Stratagene Mx 3005P System. The assay was a SYBR Green-based real-time PCR assay[15] performed using a QuantiTect SYBR Green PCR Kit (Qiagen).

The primers used were: forward-CACGCTGTTTTGACCATAGAAGACAC; reverse-TTCTTTGATTTGCACCACCGGATCC. The qPCR reaction was performed under the following thermal cycling conditions: 95 °C for 15 minutes; 40 cycles of 95 °C for 15 seconds, 60 °C for 30 seconds and 72 °C for 30 seconds; and 10 minutes at 72 °C for extension, followed by a melting curve analysis.

Prior to being used for study sample analysis, the qPCR assay was validated at LRRI for accuracy, precision, specificity, LLOQ, standard curve, and sample stability. Tissues analyzed using 0.5 μg DNA per reaction included liver, adrenal gland, brain, heart, testis/kidney, ovary, spleen, and bone marrow. The LLOQ of the assay was determined to be 35 vg/μg genomic DNA using 0.5 μg of genomic DNA per reaction. Tissues analyzed using 0.1 μg DNA per reaction were knee, popliteal lymph nodes, lungs, and blood. The LLOQ was determined to be 50 vg/μg genomic DNA using 0.1 μg of genomic DNA per reaction.

Analysis of human and rat IL-1Ra protein level in knee joints and serum

Rat and human IL-1Ra protein expression was measured in the knee joints of the satellite animals. Knee tissues were collected at necropsy and stored frozen at −80 ± 10 °C. Upon analysis, each frozen knee tissue sample was weighed and then homogenized using a "Tissuemizer" (Tekmar, Mason, OH) in Dulbecco's phosphate-buffered saline (Cat # D8537, Sigma-Aldrich) containing 0.1% Triton X-100 (Cat. # T8787, Sigma-Aldrich) and a cocktail of proteinase inhibitors (Cat. # P8340, Sigma-Aldrich). Rat IL-1Ra expression was analyzed in groups 1–4 at all the time points, and human IL-1Ra was analyzed in group 5 at SD 26. The serum rat and human IL-1Ra levels were determined in both the main and satellite animals. The IL-1Ra content was analyzed using commercially purchased ELISA kits according to standard procedures developed based on the manufacturer's instructions. The rat IL-1Ra assay used a modification of the DuoSet kit for mouse IL-1Ra (Cat# DY480, R&D Systems, Minneapolis, MN) with rat IL-1Ra used to prepare the standard curves. The human IL-1Ra assay used a DRA00B ELISA kit (R&D Systems). The ELISA assays for both rat IL-1Ra and human IL-1Ra were qualified prior to being used for study sample analysis at LRRI. The LLOQ for the rat IL-1Ra assay was 391 pg/ml and for the human IL-1Ra protein assay was 31.3 pg/ml. The IL-1Ra protein levels in the knee joints were normalized to the total protein content (Pierce Coomassie Protein Assay Kit, Thermo Fisher) of the homogenates.

IFN-γ ELISpot analysis of splenocytes

T-cell responses to the vector capsid were analyzed using splenocytes obtained from rats euthanized on SD 26 and SD 180. At necropsy, a portion of the spleen was collected and used to prepare single cell suspensions. The splenocyte suspensions were frozen in freezing media (90% fetal bovine serum/10% dimethylsulfoxide) at −80 ± 10 °C and then transferred to a liquid nitrogen cryogenic freezer for storage until ELISpot analysis.

The IFN-γ ELISpot assays were performed using a commercial assay kit (Cat# 3220-2H, Mabtech, Mariemont, OH) according to the manufacturer's instructions. Three peptide pools (*i.e.*, pp1, 2, and 3) of 80–110 peptides each were designed as 15-mers offset by five amino acids and spanning the AAV2.5 capsid protein VP1 (736 amino acids). The peptides were synthesized by ProImmune (Sarasota, FL). The spleen cells (2.5×10^5 viable cells) were stimulated with pp1, pp2, or pp3, and the IFN-γ–producing cells were detected and counted on an Immunospot plate reader (ImmunoSpot S5 Macro Analyzer with ImmunoSpot Software from Cellular Technology, Shaker Heights, OH). Each cell sample was run in duplicate. Along with peptide pool stimulation, a subset of cell samples were also stimulated with cell culture media (media control stimulus), dimethyl sulfoxide (diluent control stimulus), or concanavalin A (positive mitogenic stimulus). In addition, on each day of analysis, negative control cells (naive rat splenocytes) and positive control cells (splenocytes collected from rats administered 5×10^{10} vg of the rat vector sc-rAAV2.5rIL-1Ra by i.m. injection on day 42 post-injection) were also included for quality control purposes.

Titration of anti-AAV2.5 NAb in serum

The sera collected at necropsy from group 1–5 main study rats and group 6 rats were assayed for NAb to the AAV2.5 vector at the University of Pennsylvania Immunology Core. The assay was validated prior to sample analysis. The serum samples were heat-inactivated at 56 °C for 35 minutes. Recombinant AAV2.5.CMV.LacZ (10^9 genome copies/well, University of Pennsylvania Vector Core) was diluted in serum-free Dulbecco's Modified Eagle medium (Corning Cellgro, Tewksbury, MA) and incubated with twofold serial dilutions (starting at 1:5) of heat-inactivated serum samples in Dulbecco's Modified Eagle medium for 1 hour at 37 °C. Subsequently, the serum–vector mixtures were added to 96-well plates seeded with Huh7 cells at 1×10^5 cells/well that had been infected with wild-type human adenovirus type 5 (wtHAdV5, 45 particles/cell) 2 hours earlier. After 1 hour, each well was supplemented with an equal volume of 20% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA) in Dulbecco's Modified Eagle medium and incubated for 18–22 hours at 37 °C and 5% CO_2. Then, the cells were washed twice in PBS (Corning Cellgro, Tewksbury, MA) and lysed. The lysate was developed with mammalian β-Galactosidase Assay Kit for bioluminescence according to the manufacturer's protocol (Applied Biosystems, CA) and measured in a microplate luminometer (Clarity, BioTek). The NAb titer was reported as the highest serum dilution that inhibited AAV2.5.CMV. LacZ transduction (β-gal expression) by 50% or more as compared with the mouse serum control (Sigma, Allentown, PA).

Statistical analysis

The analyses of the feed consumption, terminal body weight, and organ weight data were performed using the Tables & Statistics module of the Provantis software (the statistics are SAS software-based). One-way analysis of variance (ANOVA) was used to compare the vehicle control and vector dose groups, and if significant, Dunnett's test was used to compare each vector dose group to the vehicle control.

The statistical analyses of the clinical pathology and in-life body weight data were performed using one-way ANOVA to assess treatment effects and two-way ANOVA (treatment, time, treatment × time) to assess treatment effects across time. If the ANOVA (one- or two-way) indicated significant difference among dose groups, Dunnett's multiple comparison test was applied to assess differences between each vector-dosed group and the vehicle control group. The analyses were performed using the SAS software system, version 9.2 (Cary, NC). Statistical analyses of the ELISA data and ELISpot data were performed using GraphPad Prism (La Jolla, CA). *T*-test (for two groups), one-way ANOVA (for treatment effects more than two groups), and two-way ANOVA (for treatment and stimuli effects for ELISpot data) analyses were performed as appropriate for the data. All statistical significance was assessed at $P \leq 0.05$.

CONFLICT OF INTEREST

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