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Effective reduction of the interleukin-1β transcript in osteoarthritis-prone guinea pig chondrocytes *via* short hairpin RNA mediated RNA interference influences gene expression of mediators implicated in disease pathogenesis

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summary

Objective—To ascertain a viral vector-based short hairpin RNA (shRNA) capable of reducing the interleukin-1β (IL-1β) transcript in osteoarthritis (OA)-prone chondrocytes and detect corresponding changes in the expression patterns of several critical disease mediators.

Methods—Cultured chondrocytes from 2-month-old Hartley guinea pigs were screened for reduction of the IL-1β transcript following plasmid-based delivery of U6-driven shRNA sequences. A successful plasmid/shRNA knockdown combination was identified and used to construct an adeno-associated virus serotype 5 (AAV5) vector for further evaluation. Relative real-time reverse transcription polymerase chain reaction (RTPCR) was used to quantify *in vitro* transcript changes of IL-1β and an additional nine genes following transduction with this targeting knockdown vector. To validate *in vitro* findings, this AAV5 vector was injected into one knee, while either an equivalent volume of saline vehicle (three animals) or non-targeting control vector (three animals) were injected into opposite knees. Fold differences and subsequent percent gene expression levels relative to control groups were calculated using the comparative CT ($2^{-\Delta\Delta CT}$) method.

Results—Statistically significant decreases in IL-1β expression were achieved by the targeting knockdown vector relative to both the mock-transduced control and non-targeting vector control groups *in vitro*. Transcript levels of anabolic transforming growth factor-β (TGF-β) were significantly increased by use of this targeting knockdown vector. Transduction with this targeting AAV5 vector also significantly decreased the transcript levels of key inflammatory cytokines [tumor necrosis factor-α (TNF-α), IL-2, IL-8, and IL-12] and catabolic agents [matrix metalloproteinase (MMP)13, MMP2, interferon-γ (IFN-γ), and inducible nitrous oxide synthase (iNOS)] relative to both mock-transduced and non-targeting vector control groups. *In vivo* application of this targeting knockdown vector resulted in a >50% reduction (P= 0.0045) or >90% (P= 0.0001) of the IL-1β transcript relative to vehicle-only or non-targeting vector control exposed cartilage, respectively.

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Conclusions—Successful reduction of the IL-1β transcript was achieved *via* RNA interference (RNAi) techniques. Importantly, this alteration significantly influenced the transcript levels of several major players involved in OA pathogenesis in the direction of disease modification. Investigations to characterize additional gene expression changes influenced by targeting knockdown AAV5 vector-based diminution of the IL-1β transcript *in vivo* are warranted.

Keywords

Osteoarthritis; Interleukin-1β; Chondrocytes; Guinea pig; Adeno-associated virus vector; RNA interference

Introduction

Multiple molecular and biomechanical factors induce signaling cascades that lead to extracellular matrix (ECM) destruction and osteoarthritis (OA). Specifically, interleukin-1β (IL-1β) is considered a primary instigator of OA[1]. In cell culture and explants, IL-1β inhibits maintenance of the ECM network[2] and directly provokes protease-induced cartilage destruction[3]. IL-1β is also a mediator of inflammation and may be involved with pain mechanisms in joints affected by OA[4]. Thus, it is recognized that inhibiting the biological activities of IL-1β could modify or alleviate the disease at several levels[5].

In vitro and *in vivo* efforts to reduce or block IL-1β have been demonstrated using diacerein/rhein or IL-1 receptor antagonist protein (IRAP) in experimental OA[4,6–18]. Although these studies suggest that IL-1β is important for the development of secondary OA, IL-1β knockout mice show accelerated progression of OA lesions compared to wild-type litter mates[1]. Use of knockout mice to study the pathogenesis of OA is valuable but suffers from drawbacks, including potential for compensatory gene expression[1]. Alternatively, RNA interference (RNAi) technology can be used to generate loss-of-function phenotypes without complications associated with traditional knockout animals[19]. Targeted, localized reduction of gene products implicated in naturally-occurring OA provides the opportunity to define contributions of specific molecular pathways to the disease and offers potential as a therapeutic strategy[20]. Further, delivery of small RNAs capable of inciting cellular knockdown machinery *via* lowly immunogenic and persistent viral vectors, such as recombinant adeno-associated virus (AAV), have emerged as capable systems for application in musculoskeletal disorders[21]. AAV-based delivery of RNAi constructs has been successful in cell culture and animal model systems[20] and holds promise in the context of OA.

Currently, there are minimal reports using RNAi to study the pathogenesis of OA. It was our hypothesis that reducing the IL-1β transcript *via* delivery of a plasmid-based and, subsequently, an AAV vector-based short hairpin RNA (shRNA) sequence would alter expression patterns of additional mediators implicated in OA. Importantly, this knockdown was investigated, and sustained, with and without inflammatory insults instigated by either lipopoly-saccharide (LPS) or exogenous recombinant human (rh)IL-1β. Moreover, evidence is provided that reduction of IL-1β may be plausible *in vivo* using this validated tool.

Materials & methods

All procedures were approved by the university's Institutional Laboratory Animal Care and Use Committee and performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Cartilage was aseptically harvested from knees of five 2-month-old male Hartley guinea pigs (Charles River Laboratories, Wilmington, MA), pooled, and minced. Cells were isolated following a 6-hour digestion in

0.2% collagenase I (Gibco, Grand Island, NY) and expanded in standard culture conditions in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 29.2 mg/ml L-glutamine, 50 U penicillin/ml, and 50 U streptomycin/ml until >95% confluent. Chondrocytes were implemented prior to their fourth passage. Six 2-month-old male animals were used for the *in vivo* study.

Plasmid screening

Experiments to screen plasmids containing potential knockdown shRNA sequences were performed in triplicate. The selected plasmid allowed simultaneous expression of shRNA *via* the U6 promoter and CMV-driven enhanced green fluorescent protein (GFP) expression [Fig. 1(A)] and is intended for making single-stranded AAV vectors. Ten shRNA knockdown sequences and one non-targeting sequence [Fig. 1(A)] were conceived using RNAi Block-IT Designer (Invitrogen, Carlsbad, CA) and cloned into plasmids using standard techniques. Chondrocytes (2.5×10^5) were seeded into T25 flasks (Greiner Bio-One, Monroe, NC) and allowed to reach ~50% confluency, at which time cultured cells were transfected using FuGENE®6 Transfection reagent (Roche Applied Science, Indianapolis, IN) in serum- and antibiotic-free DMEM according to recommendations at a reagent:DNA ratio of 3:1. Media containing transfection complexes were removed 24-h post-exposure; new non-supplemented Minimum Essential Media, Eagle (MEM-E) (Mediatech, Herndon, VA) was added, either with or without 1 μg/ml LPS (*Escherichia coli* O111:B4; Sigma–Aldrich, St. Louis, MO) for an additional 24-h. Cells were monitored for onset of GFP using fluorescent microscopy. All treatment groups were removed from culture flasks 48-h post-transfection for flow cytometry. Once a successful plasmid/shRNA knockdown sequence combination was identified, culture experiments to detect additional transcript changes were performed in triplicate.

In vitro knockdown

The selected plasmid/shRNA knockdown sequence combination (shRNA#1) and non-targeting control plasmid were used to produce IL-1β knockdown and non-targeting control AAV serotype 5 (AAV5) vectors, respectively, by the Viral Vector Core at Nationwide Children's Hospital (Columbus, OH) using the standard triple-transfection method[22]. Vector titration to determine DNase-resistant particles (DRP) of each vector was performed as described using quantitative real-time PCR[23]. Chondrocytes were seeded and cultured, as above, until ~75% confluent, at which time triplicate culture replicates were exposed to one of three treatments: (1)mock-transduction procedure, no vector control (C); (2) non-targeting vector control (NTC); or (3) targeting knockdown targeting knockdown vector (TV). In all groups, cells were exposed to vectors (or an equivalent volume of media for C) at a cell:DRP titer of 1:100,000 in serum-free DMEM containing 2 μM doxorubicin hydrochloride (Calbiochem, La Jolla, CA) for 2-h, followed by addition of 10% FBS[24]. Preliminary work identified this protocol and cell:DRP titer ratio as the lowest and most efficacious method able to achieve >75% transduction efficiency. After a total of 8 h, media was removed and replaced with supplemented DMEM. Cells were monitored for onset of GFP, which began 48-h post-vector exposure, using fluorescent microscopy and flow cytometry. To evaluate gene expression changes with and without inflammatory insults, cells were exposed to one of three media conditions 24-h prior to harvest: (1) fresh MEM-E, only; (2) LPS in MEM-E, as described; or (3) 10 ng/ml rhIL-1β (Roche, Mannheim, Germany) in MEM-E. All treatment groups were collected from culture flasks 48-h post-onset of GFP [Fig. 2(A)] to allow adequate time for transcript knockdown to occur.

In vivo knockdown

Three 2-month-old male Hartley guinea pigs aseptically received TV (1×10^{12} DRP in 100 μl of phosphate buffered saline (PBS)) in one knee, while the opposite knee was injected

with an equivalent volume of PBS. An additional three guinea pigs received TV in one knee or an identical vector dose of NTC in the other knee. Administered vector doses were consistent with other peer-reviewed manuscripts[25,26]. Two months post-injection, animals were humanely euthanized; cartilage was collected from the patella and patellar surface of the femur for RNA extraction to detect IL-1β transcript expression, as described below. One-step reverse transcription polymerase chain reaction (RT-PCR) (Invitrogen, Carlsbad, CA) confirmed GFP expression using specific primers (Table I; Eurofins MWG Operon, Huntsville, AL); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a positive control. Universal cycling parameters were employed for cDNA construction and amplification. Products were run in a 1% agarose gel containing ethidium bromide and visualized under ultraviolet (UV) light.

Flow cytometry

Chondrocytes were analyzed for and, for plasmid-transfected cells, separated by GFP using the i-Cyt Reflection (i-Cyt, Champaign, IL). Green fluorescence was excited by an argon 488 nm laser and detected using a 520/30 nm bandpass filter on the 520 parameter; positive cells were collected for analysis.

Relative real-time (q)RT-PCR

Total RNA was isolated from *in vitro* and *in vivo* chondrocytes *via* the NucleoSpin® RNA XS kit (Clontech, Mountain View, CA), which includes DNase treatment. cDNA was made using Taqman® Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) and qRT-PCR, complete with dissociation curve, was performed in triplicate for each gene of interest under each triplicate experimental condition (triplicate assay values for triplicate culture samples for *in vitro* work; triplicate assay values for $N = 6$ animals) using Power SYBR® Green PCR Master Mix (Applied Biosystems, Warrington, UK) and guinea pig specific primers (Table I) on the ABI Prism® 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) according to published standards and recommendations[27]. 18s ribosomal RNA and GAPDH were used as separate endogenous controls to which each gene of interest was normalized. Results were consistent between housekeeping genes; GAPDH comparisons are represented. To confirm absence of amplification, reactions were repeated using larger starting amounts of cDNA. Fold changes and subsequent percent gene expression levels relative to designated control groups were calculated using the comparative CT ($2^{-\Delta\Delta CT}$) method. For plasmid-transfection analyses, potential targeting shRNA plasmids of interest were compared to the non-targeting shRNA control sequence within each media. To stringently evaluate TV, all comparative CT values were normalized relative to C within each media (MEM-E, LPS in MEM-E, or rhIL-1β in MEM-E). *In vivo* comparative CT values for TV were compared to control knee cartilage receiving saline-only or NTC injection.

Statistical analysis

In vitro (triplicate assay values for triplicate culture samples) and *in vivo* (triplicate assay values for $N = 6$ animals) ΔCT values for each gene of interest under each experimental condition were analyzed for – and passed – Gaussian distribution and homogeneous variance using the Kolmogorov–Smirnov normality test[28]. Flow cytometry and relative percent expression qRT-PCR data (directly converted from fold changes) were expressed as mean ± 95% confidence interval. For *in vitro* data, relative qRT-PCR data within and between media conditions were analyzed for each individual gene of interest by one-way analysis of variance (ANOVA) for comparison among groups (C, NTC, and TV) followed by pairwise comparisons using Tukey 95% confidence intervals. Paired *t*-tests were utilized for *in vivo* comparisons within C/TV and NTC/TV groups; statistical differences in ΔCT values were not detected within or between animals. All analyses were performed using the

Minitab statistical software program (State College, PA) with a statistical significance of $P < 0.05$.

Results

Preliminary studies to detect IL-1β in 2-month-old guinea pig chondrocytes at transcript and protein levels *via* RT-PCR and immunohistochemistry, respectively, confirmed presence in host tissue and cultured cells up to the fourth passage [Fig. 1(B)]. Flow cytometry revealed that 28.15± 2.44% of plasmid-transfected cells were positive for GFP [Fig. 1(C)], which is consistent with other lipid-based DNA delivery methods for primary chondrocytes[29]. Relative qRT-PCR identified three of ten shRNA sequences that significantly decreased IL-1β transcript levels relative to the non-targeting control plasmid [Fig. 1(D)]. In control media, shRNA #1 achieved a degree of knockdown (89.42± 0.38%) that was greater ($P = 0.004$) than the reduction provided by shRNA#2 and #3 and was anticipated to influence expression of other genes of interest. This knockdown (97.18±2.72%) was maintained ($P = 0.002$) during active LPS challenge [Fig. 1(D)].

The influence of IL-1β knockdown on expression of other mediators was comparable between plasmid-transfected and vector-transduced chondrocytes; expression changes for the latter are presented.

In vitro RNAi

Flow cytometry on pilot studies revealed that 87.50± 4.55% of chondrocytes expressed GFP 48 h following initial 2-h application of AAV5 vectors [Fig. 2(A)]. Amplification was not detected for the following transcripts (Table I): collagenase I/matrix metalloproteinase-1 (MMP1); IL-4; IL-6; and IL-10 (data not shown).

IL-1β transcript

Media influence—Exogenous LPS increased ($P < 0.001$) transcript expression of IL-1β relative to control media (Table II). Exogenous rhIL-1β, however, decreased ($P = 0.007$) expression of IL-1β relative to control media (Table II), and treatment with either AAV5 vector significantly reduced this expression further (Fig. 2).

Transcript changes—Significant decreases in IL-1β expression were achieved by TV relative to C and NTC groups in all media [Fig. 2(B)]. Specifically, IL-1β was reduced to 48.00 ±2.54%, 37.25 ±1.44%, and 6.50 ± 0.29% relative percent expression levels in control, LPS, and rhIL-1β media, respectively. This knockdown is particularly striking given that NTC increased ($P < 0.001$) transcript levels of IL-1β in control and LPS media. Although exogenous rhIL-1β statistically decreased IL-1β expression (which was further exaggerated in the presence of AAV5 vector), TV continued to result in a decreased IL-1β transcript level relative to C ($P < 0.001$) and NTC ($P = 0.03$) control groups.

Inflammatory mediators

Media influence—Overall, exogenous LPS and rhIL-1β significantly increased transcript expression of inflammatory genes of interest relative to control media (Table II). IL-2, however, was only detected in media containing rhIL-1β [Fig. 3(B)] and LPS media reduced expression of IL-12 to below detection limits [Fig. 3(D)].

Influence of RNAi—Transcript changes associated with inflammatory agents were significantly affected following exposure to TV and NTC, and these changes were often influenced by ambient media that was present. In general, transduction with TV statistically

decreased transcript levels of key inflammatory cytokines relative to C and NTC groups. Findings relevant to individual genes and media are elucidated below.

Tumor necrosis factor-α (TNF-α)—In control and rhIL-1β media, TNF-α transcript levels were significantly reduced by TV relative to C and NTC control groups [Fig. 3(A)]. In rhIL-1β media, TNF-α was decreased ($P = 0.002$) by NTC relative to C, but reduction continued to be most significant by TV. In LPS media, however, TNF-α was increased ($P < 0.001$) in TV and NTC groups compared to C. Further, TNF-α was greater ($P < 0.001$) in TV treated cells than NTC-treated cells.

IL-2—IL-2 was detected only in rhIL-1β media treatment groups [Fig. 3(B)]. IL-2 was increased ($P < 0.001$) by TV compared to NTC and C control groups.

IL-8—Expression patterns observed for the IL-8 transcript were influenced by media conditions [Fig. 3(C)]. In control media, IL-8 levels were increased ($P < 0.001$) by NTV relative to TV and C. No statistical difference was found between the latter two treatments. In LPS media, however, TV and NTC groups were increased ($P < 0.001$) relative to C. Exogenous rhIL-1β media, on the other hand, decreased ($P < 0.001$) the IL-8 transcript in the presence of either AAV5 vector, with a significant reduction ($P = 0.02$) induced by TV.

IL-12—IL-12 was present in control and rhIL-1β media, only, and was statistically lower in TV relative to C and NTC control groups in both conditions [Fig. 3(D)]. In control media, IL-12 was increased ($P < 0.001$) in NTC relative to C, while it was decreased ($P = 0.002$) by NTC relative to C in the presence of rhIL-1β.

Control media—In general, transcript levels of inflammatory cytokines in control media were either below detection or demonstrated a significant decrease in the presence of TV (Fig. 3). Interestingly, IL-8 and IL-12 were significantly increased when transduced by NTC relative to C.

LPS media—In LPS media, IL-2 and IL-12 were below detection limits. TNF-α [Fig. 3(A)] and IL-8 [Fig. 3(C)], however, showed increased ($P < 0.001$) cytokine transcript expression in the presence of either the TV or NTC AAV5 vector. TNF-α, in particular, was further increased ($P < 0.001$) in TV treated cells exposed to LPS media relative to NTC cells.

rhIL-1β media—All transcripts of interest, except for IL-2, were statistically decreased by TV in exogenous rhIL-1β media (Fig. 3). For IL-2, transduction with TV resulted in an increase ($P < 0.001$) in IL-2 transcription relative to C and NTC control groups [Fig. 3(B)].

Anabolic and catabolic agents

Media influence—Overall, exogenous LPS and rhIL-1β significantly decreased transcript expression of transforming growth factor-β (TGF-β) relative to control media (Table II). LPS and rhIL-1β treatment, however, statistically increased transcript numbers of all catabolic agents investigated (Table II). Gelatinase A/MMP2 was only detected in control media following exposure to NTC [Fig. 5(B)].

Influence of RNAi—In general, transcript levels of anabolic TGF-β were significantly increased by use of TV relative to C and NTC control groups. Transduction with TV also statistically decreased transcript levels of catabolic agents relative to C and NTC. Results pertinent to individual genes and media are discussed below.

TGF-β—In LPS and rhIL-1β media, expression of TGF-β was significantly increased by TV relative to NTC (Fig. 4). Of note, TV resulted in an increase ($P < 0.001$) in TGF-β relative to C in LPS media. In control media, both AAV5 vectors statistically increased TFG-β transcript levels relative to C; a significant difference between these two AAV5 vectors was not present.

Collagenase III/MMP13—In all media, MMP13 was significantly reduced following transduction with TV [Fig. 5(A)]. In contrast, MMP13 was statistically increased by NTC relative to C in control and LPS media. In rhIL-1β media, MMP13 was decreased in both AAV5 vector treatment groups relative to C, but TV continued to demonstrate fewer ($P = 0.03$) MMP13 transcripts relative to NTC.

Gelatinase A/MMP2 and interferon-γ (IFN-γ)—Expression patterns for MMP2 and IFN-γ were similar. In both cases, gene expression was significantly decreased by TV relative to C and NTC control groups in LPS and rhIL-1β media [Fig. 5(B and C)]. Further, the IFN-γ transcript was reduced ($P < 0.001$) by TV relative to C in control media. While NTC statistically decreased transcript levels relative to C in LPS-treated chondrocytes, MMP2 and IFN-γ transcripts were significantly increased by NTC in rhIL-1β media.

Inducible nitrous oxide synthase (iNOS)—In control and rhIL-1β media, iNOS was below detection limits following treatment with TV [Fig. 5(D)]. For LPS media, iNOS was not different between TV and C groups; transcript levels, however, were significantly higher ($P < 0.001$) in the NTC group. In all media, transduction with NTC resulted in statistically increased expression levels of iNOS relative to TV and C.

Control media—In control media, treatment with either AAV5 vector resulted in a statistical increase in TGF-β transcript numbers (Fig. 4). With the exception of MMP2, for which expression was only detected following NTC treatment, all catabolic genes were significantly reduced by TV relative to C and NTC (Fig. 5). Of note, transcript numbers for all catabolic genes were increased when transduced by NTC relative to C.

LPS media—In LPS media, expression of TGF-β was increased ($P < 0.001$) by TV relative to C and NTC (Fig. 4). Except for iNOS, transcript levels of all catabolic agents in this media were either below detection or demonstrated statistical decreases following exposure to TV (Fig. 5).

rhIL-1β media—Treatment with either AVV5 vector resulted in significantly reduced TGF-β transcript numbers relative to C (Fig. 4). Importantly, expression of TGF-β was decreased ($P = 0.04$) by TV relative to NTC in this media. Transcript levels of all catabolic agents following exposure to TV demonstrated statistical reductions relative to C and NTC (Fig. 5). Except for MMP13, remaining catabolic mediators were also significantly increased by NTC relative to C.

In vivo RNAi—A decrease ($P = 0.0045$) in IL-1β transcript level (44.25 ± 8.16%) was attained in cartilage *in vivo* by TV relative to PBS-only, contralateral control knees [Fig. 6(A)]. A reduction ($P = 0.0001$) in IL-1β (5.50 ± 8.00%) was also present in cartilage exposed to TV relative to contralateral NTC-treated knees. Importantly, reporter gene expression was detected in cartilage harvested from knees injected with TV or NTC [Fig. 6(B)].

Discussion

We recently described the temporal expression and tissue distribution of IL-1β through progression of primary OA in two strains of guinea pigs with varying disease propensity[30]. Persistent IL-1β was found in cartilage, menisci, synovium, and subchondral bone in OA-prone animals at 120 and 180 days, while OA-resistant animals demonstrated a significant decrease in expression, a finding that suggested a relationship between IL-1β and premature disease incidence in the former strain. These findings prompted the current work, which had a primary aim of identifying a tool capable of reducing IL-1β-mediated pathways such that evidence of its mechanistic contribution to OA could be determined.

To our knowledge, this is the first study to demonstrate AAV5 vector-based reduction of the IL-1β transcript *via* U6-driven shRNA-mediated RNAi in OA-prone guinea pig chondrocytes. Importantly, cells *in vitro* were exposed to two standard inflammatory agents, LPS and rhIL-1β, to demonstrate persistent knockdown during biologically relevant scenarios where an upregulation of the IL-1β transcript is anticipated. This knockdown also resulted in statistically significant changes in gene expression of other factors implicated in OA pathogenesis in all media conditions. Of note, key inflammatory (TNF-α, IL-2, IL-8, and IL-12) and catabolic agents (MMP13, MMP2, IFN-γ, and iNOS) were significantly decreased by TV relative to C and NTC, while an anabolic factor (TGF-β) was statistically increased. Further, *in vivo* application of TV revealed that IL-1β levels were reduced >50% relative to contralateral control cartilage and >90% relative to NTC-exposed cartilage. A long-term study, including additional timepoints and an increased sample size, is merited to pursue associated genes of interest that may be influenced by this degree of *in situ* transcript reduction in cartilage, as well as other relevant joint tissue.

In general, transcript patterns observed following targeted reduction of IL-1β *via* RNAi in guinea pig chondrocytes coincided with findings exhibited following *in vitro* treatment with diacerein/rhein or IRAP, particularly in regards to MMPs, iNOS, and TGF-β. For example, diacerein/rhein has been shown to decrease secreted protein levels of proMMPs-1, -3, and -13 in cultured rabbit articular chondrocytes exposed to rhIL-1α at a concentration of 1 ng/ml[6]. Human OA cartilage treated with exogenous rhIRAP reduces these same mediators at the transcript level[7]. Further, in equine synoviocytes transduced with an adenoviral vector carrying a coding region for IRAP, MMP13 mRNA expression was significantly lower compared to non-transduced controls[8]. Interestingly, however, one study evaluating diacerein did not detect a significant difference in MMP13 in media of human OA chondrocytes stimulated by rhIL-1β (10 U/ml), but differences in experimental design may account for this discrepancy[9]. Reduction of iNOS mRNA and NO release from bovine chondrocytes[10], human OA chondrocytes in alginate beads[11], and LPS-stimulated (1 mg/ml) human OA cartilage[12] have also been observed following application of diacerein/rhein in a dose-dependent manner. The study utilizing human cells in alginate beads also demonstrated that, in media containing 10^{-10} M rhIL-1β, rhein did not significantly modify IL-8 production[11]. Enhanced expression of TGF-β1 and TGF-β2 mRNA and total mature TGF-β protein were observed in bovine chondrocytes treated with 10 ng/ml IL-1β (species source not indicated)[13], as well as rabbit chondrocytes[14]. Corroboration of our findings with other work provides evidence that use of vector-based shRNA to reduce the effective concentration of the IL-1β transcript in chondrocytes may be equivalent to established methods.

TNF-α in LPS media and IL-2 in rhIL-1β media were statistically increased by TV relative to C and NTC controls. Although there are no studies that have previously discussed the effects of IL-1β antagonism on IL-2 expression, a study using equine synoviocytes in basal media and media containing 10 ng/ml IL-1β demonstrated that TNF-α was significantly

increased following transduction with an adenoviral vector containing a coding region for IRAP, particularly when exposed to the latter treatment[15]. Additional work is necessary to establish mechanisms leading to increases in these inflammatory cytokines with concurrent IL-1β reduction and in relation to specific media. Our findings emphasize complex and opposing effects that ambient environmental stimulators have on gene expression in the face of identical treatment protocols.

Our study also demonstrated that NTC significantly increased transcript levels of IL-1β, TNF-α, IL-8, IL-12, MMP13, MMP2, IFN-γ, and iNOS in various media relative to C and TV. AAV, itself, is known to be a weak innate immunogen, and cytokine and chemokine responses in transduced tissues are limited and highly transient[16]. For instance, intravenous administration of an AAV vector resulted in rapid induction of TNF-α mRNA in murine liver cells that returned to normal levels within 6 h[17]. As such, the significance of inflammatory transcript changes noted in our *in vitro* study may not have relevant *in vivo* implications. The influence that a non-targeting control AAV5 vector may have on catabolic agents, however, is unknown and merits consideration.

Published qRT-PCR primer sets for MMP1, IL-4, IL-6, and IL-10 were validated in our laboratory and tested throughout this study, but did not result in detectable amplicons. These findings may, indeed, indicate that these mediators were not undergoing transcription, but further work continues to trouble-shoot assay conditions and assess alternate primer options. We were particularly interested in MMP1, as differing effects related to IL-1β antagonists have been reported. In one study, rhein reduced transcript levels of this catabolic agent in rhIL-1β (10 ng/ml) stimulated bovine chondrocytes[18], while another study using rhIL-1β (10 U/ml) treated human OA chondrocytes were exposed to diacerein and a significant reversal in MMP1 levels was not detected in culture media[9]. In contrast, exogenous hrIRAP has shown a dose-dependent decrease in MMP1 mRNA in the canine model of experimental OA[19]. Further, IL-6 production in human chondrocytes was statistically decreased by rhein in unconditioned media but was not affected by diacerein in media containing 10^{-10} M rhIL-1β[11].

Media was collected from treatment groups to correlate transcript changes to detectable protein levels. Given need to exhaustively validate reagent cross-reactivity with guinea pig epitopes, as well as account for inherent lability of several mediators[31], studies persist to correlate mRNA data with protein data *via* Western blot and/or enzyme-linked immunosorbent assay (ELISA) analysis. To date, our laboratory has tested several commercially available ELISA kits designed for use with human, mouse, and rat epitopes, but guinea pig IL-1β levels continue to be below the detection limit of these assays.

In conclusion, a plasmid- and AAV5 vector-based shRNA sequence was capable of reducing the IL-1β transcript in OA-prone chondrocytes *in vitro* with and without active inflammatory challenges. Differential gene expression of known disease mediators occurred as a result of knockdown, with influences in the direction of beneficial modification of OA pathogenesis. Importantly, *in vivo* application of TV resulted in a significant reduction of the IL-1β transcript relative to PBS-only or NTC-exposed cartilage. Investigations to characterize expression changes influenced by reduction of the IL-1β transcript *in vivo* are warranted.

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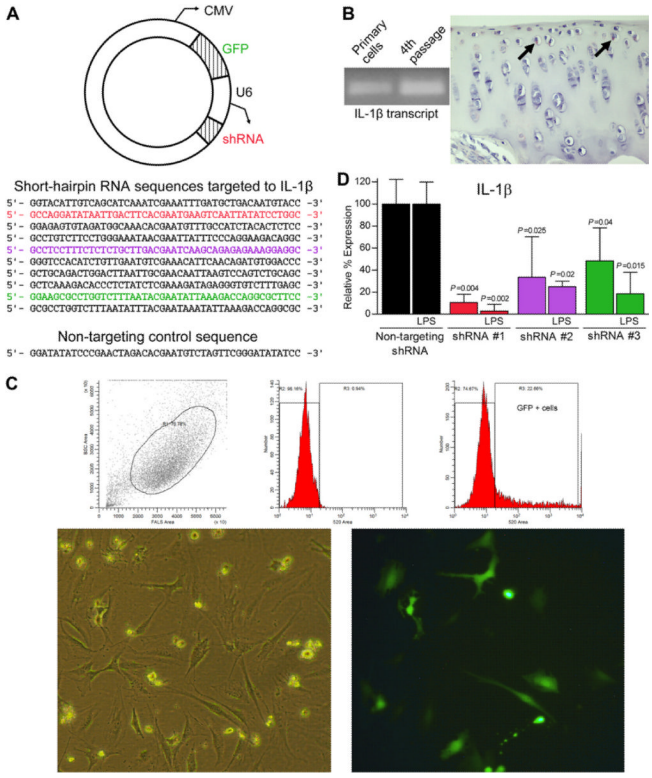

Fig. 1.

(A) Schematic representation of the AAV plasmid (top) used to deliver selected shRNA sequences (bottom) to cultured guinea pig chondrocytes. Sequences that significantly reduced IL-1β transcript expression in chondrocytes as detected by real-time RT-PCR are shown in corresponding colors in (D). (B) RT-PCR products in agarose gel (left) and positive immunohistochemistry staining (right, arrows) confirm expression of IL-1β at the transcript and protein levels, respectively, in 2-month-old primary cultured chondrocytes up to the fourth passage and *in situ* chondrocytes. (C) Representative example of flow cytometry data (top graphs) to confirm and sort GFP + chondrocytes (bottom photomicrographs,200× final magnification) in plasmid-transfected samples. (D) Significant reduction of the IL-1β transcript (mean percent gene expression levels ±95% confidence intervals) relative to a non-targeting control shRNA was detected in guinea pig chondrocytes receiving the color matched shRNA sequences in (A) delivered *via* plasmid. Within each media condition (control or LPS), fold differences and subsequent percent gene expression levels relative to C were calculated using the comparative CT ($2^{-\Delta\Delta CT}$) method. Data (triplicate assay values for triplicate culture samples) were analyzed using one-way ANOVA for comparison among groups followed by pairwise comparisons using Tukey 95% confidence intervals; significant differences are indicated.

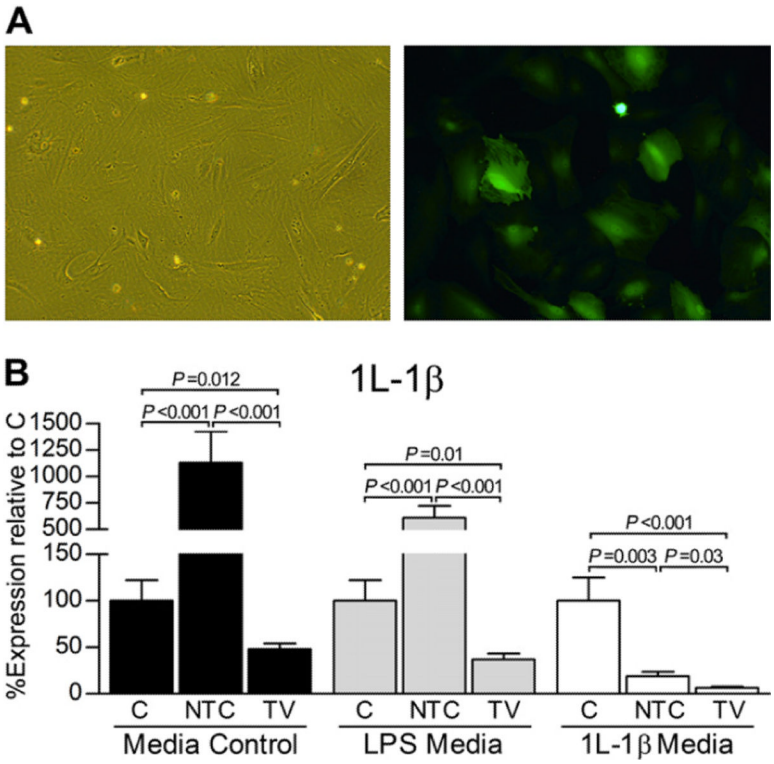

Fig. 2.

(A) Representative photomicrographs of AAV5 vector-transduced guinea pig chondrocytes (200 × final magnification). (B) *In vitro* AAV5 vector-based reduction of the IL-1β transcript via delivery of the selected shRNA #1 sequence, as indicated in Fig. 1. The targeting knockdown vector (TV) consistently demonstrated significantly reduced IL-1β transcript levels relative to the mock-transduced, no vector control (C) and the non-targeting vector control (NTC). Within each media condition (control, LPS, or IL-1β), fold differences and subsequent percent gene expression levels (mean± 95% confidence intervals) relative to C were calculated using the comparative CT ($2^{-\Delta\Delta CT}$) method. Data (triplicate assay values for triplicate culture samples) were analyzed using one-way ANOVA for comparison among groups (N, NTC, and TV) followed by pairwise comparisons using Tukey 95% confidence intervals; significant differences are indicated.

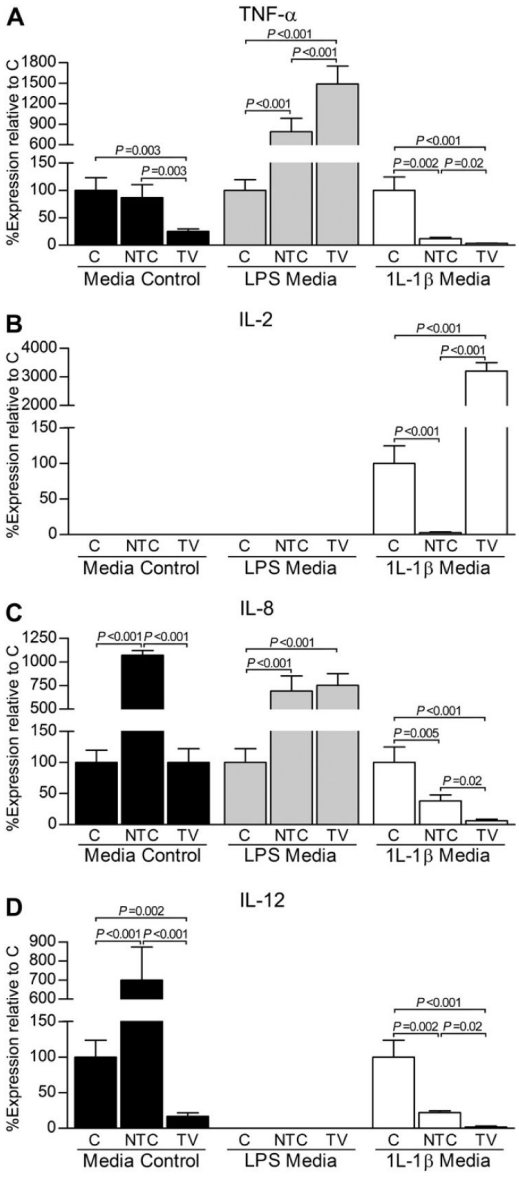

Fig. 3.

Associated transcript expression changes for inflammatory mediators TNF-α (A), IL-2 (B), IL-8 (C), and IL-12 (D) following *in vitro* AAV5 vector-based reduction of the IL-1β transcript. Within each media condition (control, LPS, or IL-1β), fold differences and subsequent percent gene expression levels (mean ± 95% confidence intervals) for the targeting knockdown vector (TV) and non-targeting vector control (NTC) relative to the mock-transduced, no vector control (C) were calculated using the comparative CT ($2^{-\Delta\Delta CT}$) method. Data (triplicate assay values for triplicate culture samples) were analyzed using one-way ANOVA for comparison among groups (N, NTC, and TV) followed by pairwise comparisons using Tukey 95% confidence intervals; significant differences are indicated.

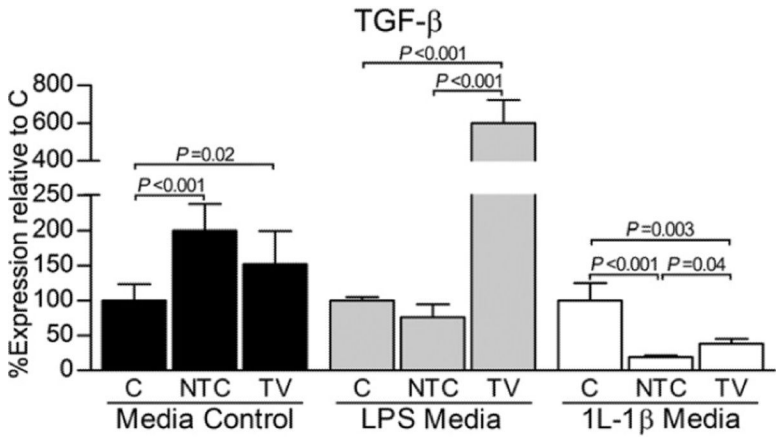

Fig. 4.

In vitro reduction of the IL-1β transcript influences transcript expression of the anabolic agent TGF-β. Within each media condition (control, LPS, or IL-1β), fold differences and subsequent percent gene expression levels (mean ± 95% confidence intervals) for the targeting knockdown vector (TV) and non-targeting vector control (NTC) relative to the mock-transduced, no vector control (C) were calculated using the comparative CT ($2^{-\Delta\Delta CT}$) method. Data (triplicate assay values for triplicate culture samples) were analyzed using one-way ANOVA for comparison among groups (N, NTC, and TV) followed by pairwise comparisons using Tukey 95% confidence intervals; significant differences are indicated.

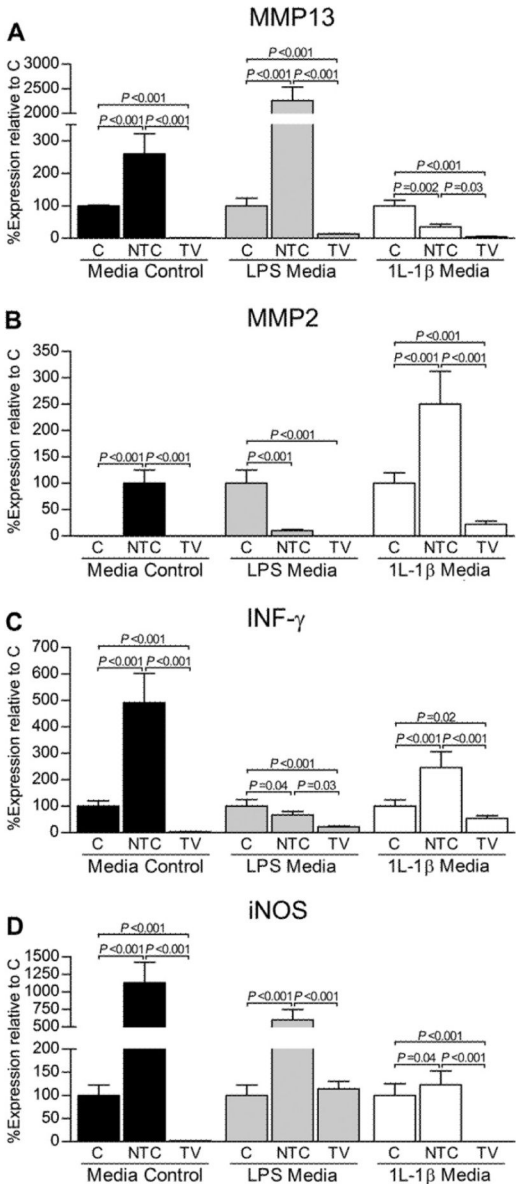

Fig. 5.

In vitro reduction of the IL-1β transcript influences transcript expression of the catabolic mediators MMP13 (A), MMP2 (B), IFN-γ (C), and iNOS (D). In general, transduction with the targeting knockdown vector (TV) significantly reduced the genes of interest in all media conditions relative to the mock-transduced, no vector control (C) and non-targeting vector control (NTC). Within each media condition (control, LPS, or IL-1β), fold differences and subsequent percent gene expression levels (mean ± 95% confidence intervals) for TV and NTC relative to C were calculated using the comparative CT ($2^{-\Delta\Delta CT}$) method. Data (triplicate assay values for triplicate culture samples) were analyzed using one-way ANOVA for comparison among groups (N, NTC, and TV) followed by pairwise comparisons using Tukey 95% confidence intervals; significant differences are indicated.

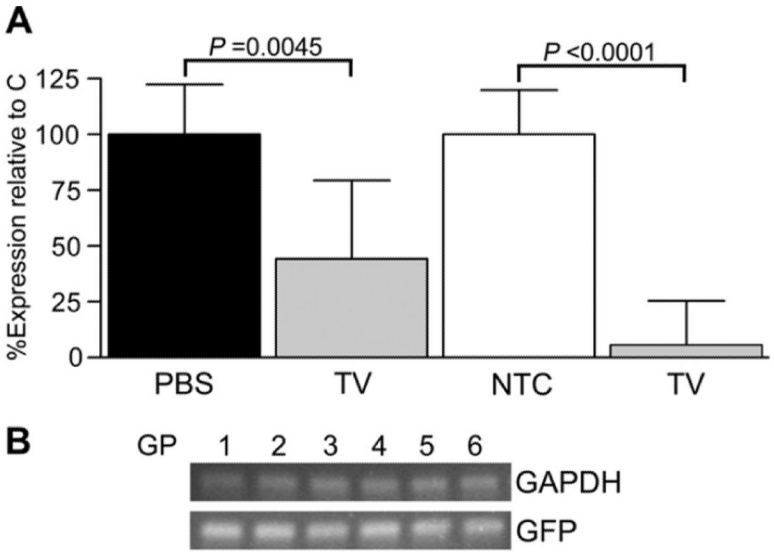

Fig. 6.

(A) *In vivo* transduction in six 2-month-old guinea pigs (GP) with the targeting knockdown (TV) AAV5 vector resulted in statistically significant reductions in IL-1β transcript expression relative to saline-only control (PBS; GP1–3) or non-targeting control vector (NTC; GP4–6) exposed cartilage in opposite knees. Fold differences and subsequent percent gene expression levels (mean ± 95% confidence intervals) for TV relative to PBS or NTC were calculated in the six animals (N = 3 in the group receiving either TV or PBS and N = 3 in the group receiving TV or NTC). For each treatment condition and individual animal, triplicate assay replicates for the gene of interest were determined via real-time RT-PCR utilizing the comparative CT ($2^{-\Delta\Delta CT}$) method and analyzed using paired t-tests; significant differences are indicated. (B) Expression of GFP was confirmed by RT-PCR in each knee injected with TV (shown) or NTC (data not shown); GAPDH served as a positive internal control.

Table I

Guinea pig specific primers used for real-time RT-PCR

Transcript of interest	Primer sequences (5′–3′)
GAPDH*	F: GTATCGTGGAAGGACTCATGACC
	R: GTTGAAGTCACAGGACACAACCT
eGFP*	F: CATGATATAGACGTTGTGGCTGTTG
	R: AAGCTGACCCTGAAGTTCATCTGC
IL-1b*	F: ACGCCTGGTGTTGTCTGAC
	R: GGGAACTGAGCGGATTC
18S ribosomal RNA†	F: TGCATGGCCGTTCTTAGTTG
	R: AGTTAGCATGCCAGAGTCTCGTT
IL-2‡	F: CTTAAGCTCTCCAAAGCA
	R: CCATCTCTTCAGAAATTCCAC
IL-4§	F: CATCGGCATTTTGAACGAGGTCA
	R: CTATCGATGAATCCAGGCATCG
IL-6§	F: CAGCCCTGAGAAAGGAGACAT
	R: AATCTGAGGTGCCCATGCTAC
IL-8†	F: GGCAGCCTTCCTGCTCTCT
	R: CAGCTCCGAGACCAACTTTGT
IL-10‡	F: GGCACGAACACCCAGTCTGA
	R: TCACCTGCTCCACTGCCTTG
IL-12‡	F: TCTGAGCCGGTCACAACTGC
	R: AGGCGCTGTCCTCCTGACAC
Collagenase 1 (MMP1)//	F: AGGTTATCCCAAAATGAT
	R: TGCAGTTGAACCAGCTATT
Aggrecanase (MMP2)§	F: AGGGCACCTCCTACAACAGC
	R: CAGTGGACATAGCGGTCTCG
Collagenase 3 (MMP13)//	F: TTCTGGCACATGCTTTTCCTC
	R: GGTTGGGGTCTTCATCTCCTG
TGF-β†	F: CATCGATATGGAGCTGGTGAAG
	R: GCCGTAATTTGGACAGGATCTG
TNF-α†	F: CCTACCTGCTTCTCACCCATACC
	R: TTGATGGCAGAGAGAAGGTTGA
IFN-γ†	F: ATTTCGGTCAATGACGAGCAT
	R: GTTTCCTCTGGTTCGGTGACA
iNOS (LT)¶	F: TGGATGCAACCCCATTGTC
	R: CCCGCTGCCCCAGTTT

* Primers designed by KSS. All amplicons were sequenced to confirm gene transcript specificity.

† Allen SS, *et al*. Infect Immun 2003;71(8):4271-7.

‡ Yamada H, *et al.* Exp Anim 2005;54(2):163-172.

§ Oh C, *et al.* Am J Obstet Gynecol 2008;199(1):78.e1-6.

[//] Huebner JL, *et al.* Arthritis Rheum 1998;41(5):877-90.

[¶] Thompson L, *et al.* Pediatr Res 2009;65(2):188-92.

Table II

ΔCT values ± Δ standard deviation (normalized to GAPDH) and corresponding percent expression (mean ± standard deviation) of transcripts of interest in inflammatory media conditions relative to control media

Transcript	Control media		LPS media		rhIL-1β media	
	ΔCT ± ΔSD	% ± SD	ΔCT ± ΔSD	% ± SD	ΔCT ± ΔSD	% ± SD
IL-1β	2.82 ± 0.12	100 ± 8.67	1.22 ± 0.10	303.41 ± 20.26 (*P*< 0.001)	3.60 ± 0.14	58.24 ± 5.39 (*P* =0.007)
TNF-α	7.22 ± 0.14	100 ± 9.38	1.20 ± 0.10	6,489.34 ± 434.57 (*P*< 0.001)	2.22 ± 0.12	3,244.67 ± 263.10 (*P*< 0.001)
IL-2	ND	–	ND	–	8.66 ± 0.14	NA
IL-8	4.62 ± 0.13	100 ± 8.72	0.11 ± 0.10	2,294.33 ± 168.74 (*P*< 0.001)	1.11 ± 0.11	1,147.16 ± 84.21 (*P*< 0.001)
IL-12	5.10 ± 0.14	100 ± 9.63	ND	–	4.70 ± 0.14	131.19 ± 11.45 (*P* =0.009)
TGF-β	−4.91 ± 0.10	100 ± 7.17	−3.50 ± 0.12	37.89 ± 3.03 (*P* =0.003)	−3.12 ± 0.10	28.71 ± 1.92 (*P* =0.002)
MMP13	2.94 ± 0.11	100 ± 7.92	−3.19 ± 0.08	7,003.48 ± 377.79 (*P*< 0.001)	−1.4 ± 0.10	2,082.15 ± 139.44 (*P*< 0.001)
MMP2	ND	–	4.32 ± 0.11	NA	6.00 ± 0.10	NA
IFN-γ	−8.12 ± 0.14	100 ± 9.25	−8.71 ± 0.02	151.57 ± 2.09 (*P* =0.005)	−9.60 ± 0.08	282.84 ± 15.26 (*P*< 0.001)
iNOS	3.16 ± 0.14	100 ± 9.34	2.42 ± 0.08	167.01 ± 9.01 (*P* =0.003)	2.22 ± 0.11	191.85 ± 14.09 (*P*< 0.001)

ND =Amplicon not detected; NA= Comparative CT($2^{-\Delta\Delta CT}$) method not applicable; *P* values are indicated.