AAV-MEDIATED DUAL-AXIS GENE THERAPY TO ENHANCE CARTILAGE REPAIR

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AAV-MEDIATED DUAL-AXIS GENE THERAPY TO ENHANCE CARTILAGE REPAIR

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The aim of this thesis project was to investigate the use of a recombinant adeno-associated virus (rAAV) as a gene therapy vector to target both the anabolic and catabolic axes of cartilage homeostasis in order to enhance cartilage repair capabilities and prevent joint degradation. The specific aims of the research were to 1) evaluate healing of full-thickness chondral defects in femoral trochlear ridges repaired with autologous chondrocytes transduced *ex vivo* with an rAAV5 vector overexpressing the anabolic protein, insulin-like growth factor I (IGF-I); 2) investigate the impact of post-transcriptional silencing of interleukin-1β (IL-1β) mediated by rAAV2 expressing a short hairpin IL-1β silencing motif in chondrocytes cultured in an osteoarthritic model and; 3) elucidate the humoral and cell-mediated immune response, and its impact on transgene expression, following direct intra-articular (IA) injection of rAAV2 and rAAV5 overexpressing IGF-I.

Autologous chondrocytes transduced *ex vivo* with rAAV5-IGF-I were arthroscopically implanted, in a fibrin vehicle, into 15mm diameter full-thickness chondral defects created in the lateral trochlear ridges of horses. Transgene expression was assessed by serially quantifying IGF-I concentration in the synovial fluid. Defect healing was assessed arthroscopically at 8 weeks post-implantation. Long-term healing at 8 months post-

implantation was assessed using gross, histological, immunohistochemical, and biochemical parameters. rAAV5-IGF-I treated defects had improved short and long-term healing with improved tissue architecture and collagen type II content compared to defects repaired with naïve chondrocytes.

Transduction of chondrocytes with rAAV2 expressing a short hairpin IL-1β led to significantly reduced IL-1β mRNA expression following stimulation with lipopolysaccharide (LPS). Down-regulation of additional key catabolic cytokines and degradative enzymes, including tumor necrosis factor (TNF)-α and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-5, was also seen in transduced cultures. Post-transcriptional silencing of IL-1β appeared to limit the catabolic cascade seen in cartilage following LPS simulation.

Direct intra-articular injection of rAAV2- and rAAV5-IGF-I led to prolonged transgene expression without any significant local joint inflammation. Carpal joints injected with rAAV5-IGF-I had significantly higher levels of IGF-I in the synovial fluid compared to rAAV2-IGF-I despite a more robust and diverse humoral immune response to AAV5. A cell-mediated immune response was not noted in either treatment group.

BIOGRAPHICAL SKETCH

Kyla Frances Ortved was born in Toronto, Canada on November 9th, 1978 to Janet May Ortved and John David Ortved. Kyla graduated from Havergal College in 1996, following which she attended the University of British Columbia where she obtained her Bachelor of Science in Animal Biology. Kyla entered veterinary school at the University of Guelph in 2002, receiving her Doctor of Veterinary Medicine degree in 2006. After graduation from the University of Guelph, Kyla completed a one year internship in large animal medicine and surgery at the University of Georgia followed by a three year residency in large animal surgery at Cornell University. Following her residency, Kyla began pursuing her Ph.D. degree in the field of Comparative Biomedical Sciences with Dr. Alan Nixon in the Comparative Orthopaedics Laboratory. She received a Graduate Research Assistantship from Cornell University and the Grayson-Jockey Club Research Foundation, Storm Cat Career Development Award during her Ph.D. training.

This dissertation is dedicated to *all* of my parents who have endlessly encouraged and supported me, and who instilled in me the courage to do what I love.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AAV	Adeno-associated virus
rAAV	Recombinant adeno-associated virus
scAAV	Self-complementary adeno-associated virus
wtAAV	Wild type adeno-associated virus
ACI	Autologous chondrocyte implantation
Ad	Adenovirus
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
CCL2	Chemokine C-C motif ligand 2
CD	Cluster of differentiation
DPBS	Dulbecco's phosphate-buffered saline
ECM	Extra-cellular matrix
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
GAG	Glycosaminoglycan
GFP	Green fluorescent protein
H&E	Haemotoxylin and eosin
IA	Intra-articular
IFN-α	Interferon alpha
IFN-γ	Interferon gamma
IGF-I	Insulin-like growth factor 1
IgG	Immunoglobulin G

IgM	Immunoglobulin M
IL-1β	Interleukin 1 beta
IL-1Ra	Interleukin 1 receptor antagonist
IL-6	Interleukin 6
IL-10	Interleukin 10
LPS	Lipopolysaccharide
MC	Middle carpal
MHC	Major histocompatibility complex
MMP	Matrix-metalloproteinase
NAb	Neutralizing antibody
NCC	Nucleated cell count
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OA	Osteoarthritis
PBMC	Peripheral blood mononuclear cells
PGE_2	Prostaglandin E_2
PFA	Paraformaldehyde
PMA	Phorbol myristate acetate
RNAi	RNA interference
sCD14	Soluble cluster of differentiation 14
sh	Short hairpin
tdT	Tandem tomato fluorescent protein
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha

TP	Total protein
qPCR	Quantitative polymerase chain reaction
vg	Viral genome

CHAPTER 1

GENERAL INTRODUCTION

Overall Goal of Dissertation Research

The overall goal of this thesis project was to investigate the impact of dual-axis gene therapy in promoting cartilage repair and preventing osteoarthritis (OA), using an adeno-associated virus (AAV) as a viral vector. In this dissertation research, the anabolic and catabolic axes of cartilage homeostasis were targeted; the effects of AAV-mediated growth factor overexpression and catabolic cytokine knockdown were investigated as potential methods of improving the repair capabilities, and limiting the degeneration, of articular cartilage. These goals were met using both *in vitro* and *in vivo* experiments in the equine model.

This introductory chapter provides pertinent background information and discussion of gene therapy for cartilage repair. The first aim of this thesis is presented in chapter 2 in which, the long-term repair of large, full-thickness chondral defects filled with autologous chondrocytes transduced *ex vivo* with an AAV vector overexpressing insulin-like growth factor (IGF-I), is investigated. IGF-I, a critical anabolic and mitogenic protein in chondrocyte homeostasis, was overexpressed in chondrocytes grafted into experimentally created defects in the lateral trochlear ridge of the femur of horses.

The second aim, presented in chapter 3, evaluates the effects of post-transcriptional silencing of IL-1β using an AAV vector expressing a short hairpin sequence targeting IL-1β. The benefit of upregulation of anabolic growth factors, such as IGF-I, is likely tempered by the known concurrent increase in catabolic factors following cartilage damage and during the subsequent degenerative process. IL-1β is arguably the most important catabolic factor in the damaged and arthritic joint; it leads to breakdown of the extra-cellular matrix (ECM) and may seriously limit the reparative effects of IGF-I. Knockdown of IL-1β was examined *in vitro* using chondrocyte monolayer and cartilage explant cultures.

The beneficial effects of gene therapy in cartilage repair were clearly demonstrated in the first two aims; however, a humoral immune response was noted in horses exposed to the AAV5 vector following *ex vivo* transduction of chondrocytes. Due to concerns regarding the effect of the humoral immune response on vector effectiveness, the final aim of this dissertation research evaluated the local and systemic humoral and cell-mediated immune response, and transduction efficiency, of two AAV serotypes injected directly intra-articularly. Understanding the immune response to AAV is vital in the development of AAV as a clinically relevant vector for gene therapy as the immune response may lead to limited transduction and transgene silencing.

Overall conclusions of this research and future directions are presented together in the final chapter.

Anatomy of Articular Cartilage

Articular cartilage is found covering bone in all diarthrodial (or movable) joints. This specialized form of cartilage, referred to as hyaline cartilage, is composed mainly of water (75%), collagen type II (15%) and proteoglycans (10%).[1] Chondrocytes, which make up only 2% of hyaline cartilage, are the only cell type in cartilage and are solely responsible for production and maintenance of the ECM, an intricate network of woven collagen type II fibrils and proteoglycans. Chondrocytes are isolated within lacunae and are surrounded by a pericellular matrix dense in decorin and aggrecan. They communicate with each other, and their environment, through soluble mediators (e.g. growth factors, cytokines) and cell adhesion molecules. Chondrocytes arise from mesenchymal progenitor cells, and following maturation their growth potential is minimal.

The ECM produced by chondrocytes is a woven matrix of collagen, proteoglycans and other glycoproteins (e.g. fibronectin) (**Figure 1.1**). The vast majority (90-95%) of collagen is collagen type II that forms homotrimer helices around collagen type XI fibrils. Procollagen is secreted by chondrocytes and is cleaved into tropocollagens extracellularly by specialized proteases prior to formation of a triple helix. Aggrecan composes 85% of the proteoglycans and consists of a core protein with covalently attached glycosaminoglycans (GAGs). Keratan sulfate, chondroitin-6-sulfate and chondroitin-4-sulfate are the main GAGs in aggrecan. The aggrecan monomer is bound to hyaluronan stabilized by link protein. Other smaller

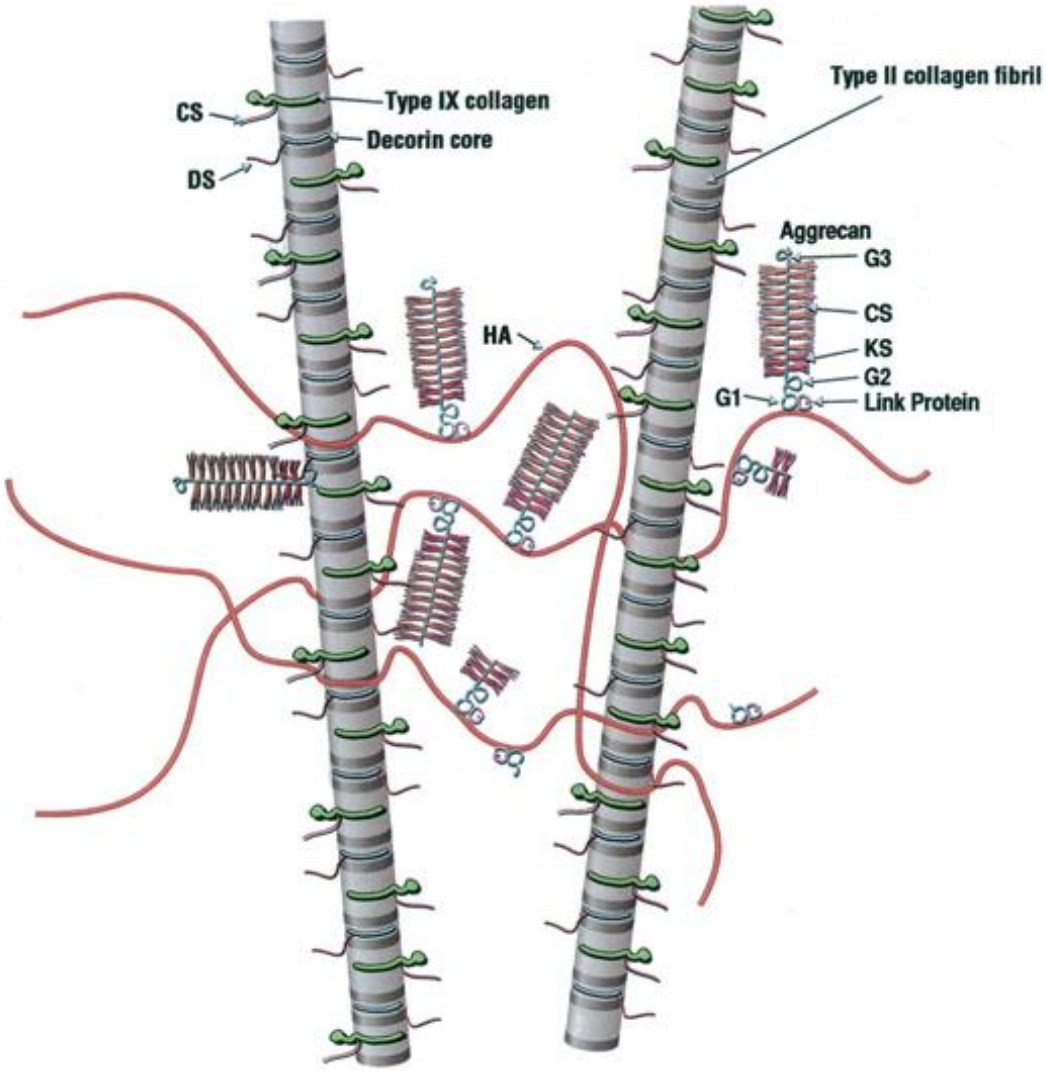

Figure 1.1 Diagram of the extra-cellular matrix (ECM) of articular cartilage including the collagen macrofibrillar network large aggregating proteoglycans (aggrecan) with hyaluronic acid. From Poole AR: Cartilage in Health and Disease. In Koopman W ed. Arthritis and Allied Conditions. A Textbook of Rheumatology. Ed 14. Vol 1. New York, Lippincott Williams & Wilkins pp 2260-2284, 2001.

proteoglycans such as decorin, fibromodulin and biglycan are found in lesser quantities within the ECM.

Articular cartilage is organized into zones defined by the specific orientation of chondrocytes and collagen fibrils, and ratio of proteoglycans to collagen (**Figure 1.2**).[1] The zonal organization of articular cartilage contributes to the biomechanical function of the tissue. The superficial (tangential) zone is defined by elongated cells and collagen fibrils that are oriented parallel to the surface. Cellular density is highest in this zone with aggrecan content being lower compared to deeper zones. The intermediate zone contains fewer cells that are more rounded, more collagen fibrils that are randomly arranged, and increased amounts of aggrecan. Finally, the deep (radiate) zone is defined by cells organized into columns parallel to the longitudinal axis of the bone. This zone has the highest aggrecan and lowest collagen content. Below the deep zone lies the tidemark, which marks the junction of non-calcified and calcified cartilage. The thin, but impenetrable, layer of calcified cartilage covers the subchondral bone.

The ECM affords the impressive biomechanical properties of articular cartilage with collagen providing tensile strength and proteoglycans providing compressive stiffness. Hyaline cartilage is also supported by dense subchondral bone, which provides additional compressive strength to the joint. The calcified cartilage layer prevents blood vessels in the subchondral bone from entering cartilage; therefore, articular cartilage is an avascular and

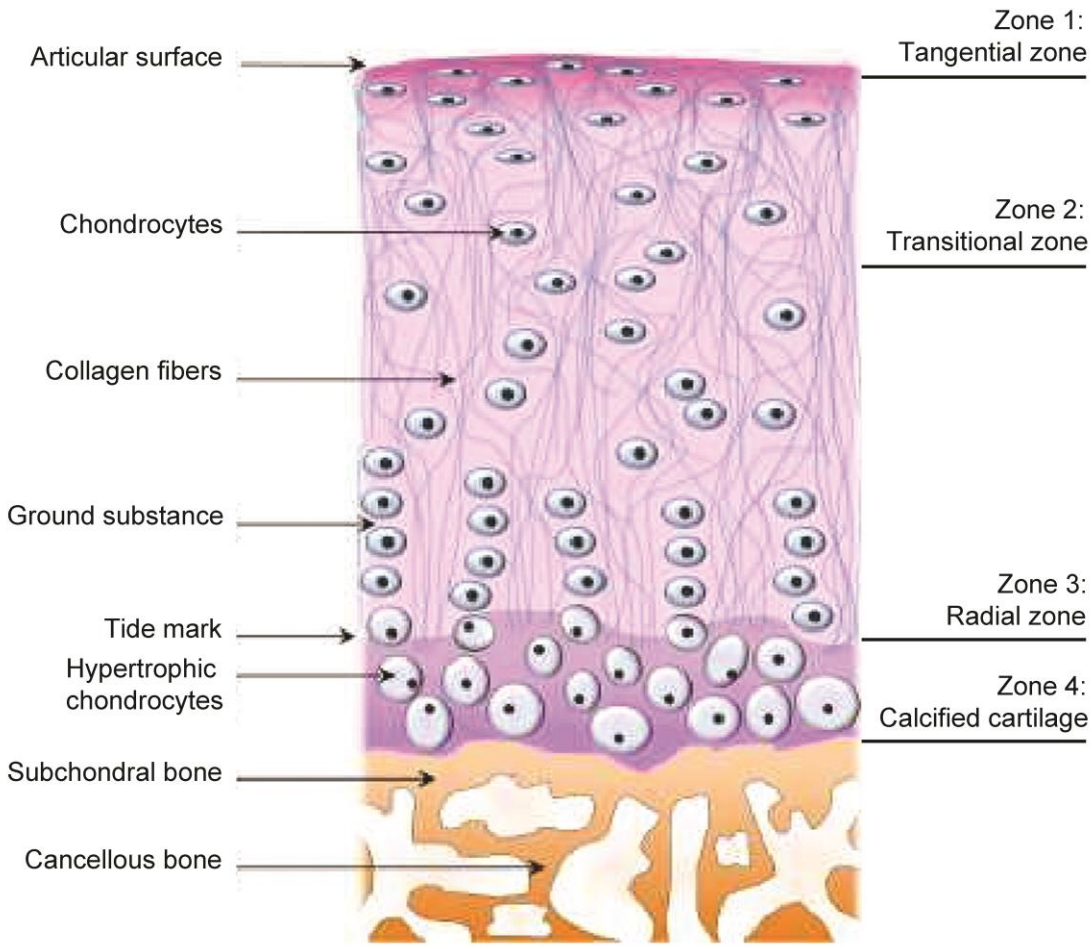

Figure 1.2 Diagram of articular cartilage depicting the zonal organization of cartilage including orientation of chondrocytes and collagen, and the relationship of cartilage to the subchondral bone. From Matta C and Zakany. Calcium signaling in chondrogenesis: implications for cartilage repair. Frontiers in Bioscience, Scholar. 5:305-324, 2013.

aneural tissue that depends on diffusion of nutrients from the synovial fluid. Due to the avascular and hypoxic nature of cartilage, chondrocytes have low metabolic rates and limited proliferative capacity. Remarkably, homeostasis of the ECM depends on the chondrocyte to balance synthesis, assembly, and degradation of matrix proteins.[1] Disruption of the physiologic balance, as seen following trauma to the articular surface and during OA, leads to dysregulated degradation of the ECM and eventual loss of a functional surface.

Joint Injury and Cartilage Repair

Articular cartilage is capable of withstanding considerable force, however, excessive trauma and injury occurs frequently, especially during athletic activity. The poor intrinsic healing capabilities of articular cartilage are due to its hypocellular, avascular and aneural nature.[2] Chondrocytes do have a limited ability to proliferate and increase ECM synthesis following injury; however, restoration of the matrix only occurs if the rate of loss does not exceed chondrocyte-mediated synthesis. Full-thickness cartilage lesions that are left untreated are filled with fibrocartilage that is biochemically and biomechanically inferior to hyaline cartilage.[3,4] Fibrocartilage is deficient in proteoglycans and collagen type II while having an over-abundance of collagen type I when compared to hyaline cartilage.[5] Due to the poor to minimal intrinsic healing capabilities of cartilage, joint injury often precipitates OA with eventual degeneration of the joint surface.[6] Methods of improving cartilage repair following injury are vital to the prevention of this debilitating disease.

Current treatment modalities to improve cartilage repair include simple debridement, enhanced intrinsic repair through microfracture or subchondral drilling, osteochondral transplantation (mosaicplasty), autologous chondrocyte implantation (ACI) and matrix-induced autologous chondrocyte implantation (MACI). Techniques for enhancing intrinsic repair include microfracture and subchondral drilling, both of which penetrate the subchondral bone plate to allow migration of mesenchymal stem cells (MSCs) and growth factors from the bone marrow to facilitate healing.[7,8] Although some benefit has been reported with these interventions, long-term outcomes have been disappointing due to apparent weakness of the repair tissue that forms. Mosaicplasty,[9,10] ACI,[11,12] and MACI[13] are all cell-based treatments in which defects are filled with autologous chondrocytes either grafted immediately from donor sites (osteochondral plugs), or harvested and expanded in the laboratory prior to grafting within a matrix (MACI) or beneath a periosteal flap (ACI). These cell-based repair strategies have shown promise for longer-term repair both in equine and human patients.[14,15] Production of a more hyaline-like repair tissue, versus fibrocartilage seen in non-cell based techniques, may explain improved treatment outcomes with these techniques.[15-17] However, regeneration of native hyaline cartilage remains challenging and genetic manipulation of chondrocytes may further enhance repair.[18] Chondrocytes can be successfully cultured in the laboratory allowing for *ex vivo* transduction. In addition, the enclosed joint offers an ideal environment for *in vivo* transduction following direct intra-articular injection of gene therapy vectors.[19]

Osteoarthritis

Osteoarthritis, a degenerative and debilitating disease, is the most common form of arthritis with more than 27 million people affected in the USA[20] and estimated annual health care costs exceeding $89 billion.[21] Following injury, chondrocytes undergo a shift in the balance between anabolism and catabolism, with decreased production of anabolic factors necessary for maintenance of the ECM and concurrent increased production of degradative cytokines and enzymes. Both acute joint injury and chronic OA are accompanied by an increase in the production of catabolic cytokines including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) produced by activated synoviocytes and chondrocytes,[22] as well as an influx of inflammatory mediators produced by leukocytes entering the joint due to increased vascular permeability. Increased production of catabolic cytokines and inflammatory mediators up-regulate destructive enzymes, including matrix metalloproteinase (MMP)-1, -3 and -13 and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-4 and -5 (aggrecanases).[23] More specifically, IL-1β has been shown to induce a cascade of pro-inflammatory mediators including MMPs and prostaglandin E2 (PGE_2)[24,25] and causes proteoglycan degradation while inhibiting collagen type II synthesis.[26,27] Overall, these alterations in the joint environment lead to disruption of cartilage homeostasis and progressive degradation of the ECM with potential for permanent damage to the articular cartilage surface.

Clinical features of OA include pain, decreased range of motion, and joint effusion. Loss of cartilage, subchondral bone sclerosis, subchondral cysts, osteophytosis and synovitis

are also common. Currently, there are no effective disease-modifying osteoarthritis drugs to halt or reverse OA. Therefore, therapeutic interventions that interfere with damaging catabolic cascades associated with cartilage degeneration and progression of OA would be of great benefit.

Growth Factors

The effects of several different growth factors on chondrocyte metabolism and cartilage repair have been investigated including insulin-like growth factor I (IGF-I),[28,29] transforming-growth factor β (TGF-β),[30,31] and bone morphogenetic protein (BMP)-2 and 7.[32,33] Amongst these growth factors, IGF-I has been identified as the critical anabolic and mitogenic protein in cartilage, with notable autocrine and paracrine effects.[34] Previous studies have shown that IGF-I increases aggrecan and collagen type II content in chondrocyte cultures, including explants, monolayer and three-dimensional systems as well as enhancing the repair potential of chondrocytes grafted into cartilage lesions in horses.[16,28] Additionally, it has been suggested that IGF-I plays an important role in the healing of damaged cartilage as it partially protects, and aids in the recovery of, the ECM following experimentally induced damage with IL-I and TNF-α.[29,35] Although chondrocyte grafts supplemented with exogenous IGF-I have been used in clinical practice,[14] the transitory nature of growth factor supplementation has led to investigation of gene therapy approaches to allow for persistent transgene expression.

Catabolic Cytokines and Degradative Enzymes

Interleukin-1β is a catabolic cytokine that is considered to be a primary instigator in the degradation of articular cartilage and development of OA.[36-38] It is a potent catabolic regulator of chondrocytes and acts to decrease proteoglycan synthesis while increasing protease-induced proteoglycan degradation.[25] More specifically, IL-1β directly down-regulates expression of collagen type II, while up-regulating expression of collagen type I and III, and inhibits aggrecan synthesis by inhibiting core protein expression.[38,39] Expression and secretion of destructive MMPs is also concurrently upregulated.[40] Additionally, IL-1β has general pro-inflammatory effects with increased expression of inflammatory mediators such as prostaglandin E2 (PGE_2)[41] and nitric oxide (NO),[42] and is likely involved with the pain mechanisms in OA joints.[43]

Proteolytic enzymes that target the macromolecules of the ECM play an important role in cartilage destruction. Both MMPs and aggrecanases (ADAMTS) are up-regulated following cartilage injury and during the arthritic process. MMP-1 (collagenase 1), MMP-3 (stromelysin 1) and MMP-13 (collagenase 3) are key players, with MMP-13 being identified as the major collagenase in the OA joint.[44] MMP-13 cleaves collagen type II, aggrecan and fibromodulin. In addition the cleaved fragments of collagen type II induce more expression of MMP-13 leading to a vicious cycle of destruction. The ADAMTS family of proteases also contributes to degradation of cartilage in OA by targeting and cleaving aggrecan.[45] There is evidence to suggest that aggrecanase mediated degradation by ADAMTS-4 and ADAMTS-5 is the predominant cause of ECM breakdown.[46]

Therapies directed towards decreasing cytokine production in the joint have been used successfully in rheumatoid arthritis.[47] Interleukin-1 receptor antagonist (IL-1RA) competitively binds the IL-1 receptor and has been shown to decrease the degradative effects of IL-1.[48] IL-1Ra therapy has been used to treat OA. However, results have been disappointing likely because the beneficial effect of IL-1Ra decreases rapidly as continued endogenous production of IL-1 alters the equilibrium to favor IL-1/IL-1R binding and re-activation.[49] Post-transcriptional silencing of IL-1β mediated by RNA interference strategies could offer a superior, more sustainable method of cytokine control.

RNA Interference Strategies

RNA interference (RNAi) was originally described in *Caenorhabditis elegans* by Fire and Mello.[50] It is a highly conserved, biologic process in eukaryotes that involves sequence-specific post-transcriptional gene silencing. mRNAs are specifically targeted by short (19-21) complementary RNA sequences that mark the transcript for degradation or prevent translation. Small RNA sequences can be endogenously produced, such as microRNA (miRNA), or exogenously introduced into the cell in the case of small interfering RNA (siRNA) or short hairpin RNA (shRNA). Regardless of the source, small double-stranded RNA (dsRNA) is cleaved by the endoribonuclease Dicer and then incorporated into the RNA-induced silencing complex (RISC). The RNA strands are separated by RISC and then the guide strand is shuttled to complementary mRNA. Following binding of the guide strand, complementary mRNA is degraded or translation is inhibited by preventing appropriate

ribosomal binding.[51,52]

The RNAi pathway is an innate cellular process that plays a large role in the regulation of gene expression, preservation of heterochromatin and maintenance of genomic stability by limiting expression of transposons.[53-55] However, the RNAi pathway can also be exploited in biological systems by specifically targeting genes to knockdown. siRNAs were originally used to achieve silencing effects. However, these molecules can be difficult to introduce into cells, have transient effects due to intracellular degradation and can elicit an innate immune response.[56] Alternatively, shRNA can be expressed by non-viral or viral vectors, allowing for increased transduction efficiency and sustained knockdown effects.[57]

Gene Therapy for Cartilage Repair

Gene therapy approaches to enhance cartilage repair and treat OA have great potential, as vectors carrying transgenes can be injected intra-articularly for concentrated, local therapeutic protein production. Gene therapy techniques, which provide long-term *in situ* expression of repair-enhancing genes, would be superior to repeated injections or depots of peptide that are transient. Additionally, injection of vectors into the discrete articular environment may avoid some of the potential systemic effects of intravascular administration. Both *ex vivo* and *in vivo* strategies have been investigated, i.e. implantation of transduced cells into the joint vs. direct intra-articular injection of gene therapy vectors. Gene transfer in the articular environment was first reported as a therapy for rheumatoid arthritis in which a retrovirus was successfully used to express IL-1Ra in human joints.[58] Although these studies

demonstrated proof-of-concept, significant safety concerns surrounded retroviral vectors due to genomic integration.[59] Other viral vectors investigated for use in OA have included lentiviral[60] and adenoviral[61] vectors due to high transduction efficiency both *in vitro* and *in vivo*. Although adenovirus has high transduction efficiency it is comparatively immunogenic, inciting significant inflammatory responses and has been associated with serious medical side effects in humans including fatalities.[62] AAV may be a more feasible and clinically relevant vector for gene therapy as it lacks pathogenicity, can invade dividing and non-dividing cells, has long-term transgene expression in animal models, and appears to be minimally immunogenic.[63]

Adeno-associated Virus

AAV is a small (20-25nm), non-enveloped, single-stranded DNA virus in the family *Parvoviridae*, genus *Dependovirus*.[63] AAV depends on a helper virus, usually adenovirus or herpesvirus, for effective replication. Wild type AAV2 (wtAAV2) is capable of site-specific integration (human chromosome 19q13.4). However, recombinant AAVs are not capable of productive infections, very rarely capable of genomic integration, and persist in the cell as concatemers, allowing for long-term transgene expression. At present there are 12 known AAV serotypes isolated from humans with more than 100 serotypes isolated from non-human primates.[63] The wild-type genome is ~5kb with 2 open reading frames: *rep* and *cap*. The *rep* gene codes 4 different replication proteins (Rep78, Rep68, Rep52, Rep40) through alternate splicing. The *cap* gene codes 3 viral proteins (VP1, VP2, VP3) that form the viral capsid. The 2 genes are flanked by inverted terminal repeats that assume a T-shaped hairpin structure.

The ITRs are the only genomic sequences required in *cis* for replication and packaging of AAV.

AAV, being a single-stranded DNA virus, requires second strand synthesis of the viral genome prior to transcription and expression of transgenes. More recently a self-complementary AAV has been developed to decrease the lag time between transduction and transgene expression. scAAV contains a dimeric inverted repeat that can fold into dsDNA without the need for second strand synthesis.[64] scAAV vectors appear to have both enhanced transduction efficiency and onset of gene expression.[65]

Recombinant AAV vectors for experimental and clinical use are generally prepared using a triple-plasmid transfection system in which 3 different plasmids are used to transfect HEK 293 cells.[66] The first plasmid, an adenovirus helper plasmid, supplies necessary Ad proteins (E1A, E1B, E4, E2A) for the helper function. The second plasmid, an AAV helper plasmid, supplies the *rep* and *cap* genes required for capsid production and viral packaging. The third plasmid, an AAV transfer plasmid, contains the transgene of interest between 2 inverted terminal repeats.

AAV has been used successfully in many preclinical studies in mice, rabbits, dogs and non-human primates and is now being used widely in clinical gene therapy trials for several diseases including hemophilia B,[67] Leber's congenital amaurosis,[68,69] and limb-girdle muscular dystrophy.[70] Tissue tropism has been shown for several AAV serotypes and these affinities are likely species-specific. AAV5 has been shown to provide efficient transgene

expression in equine chondrocytes *in vitro*. Other serotypes, including AAV2, have been evaluated for transduction efficiency of articular cells; however, preliminary data in our laboratory comparing rAAV2 and rAAV5 revealed optimal transduction of chondrocytes by AAV5 at a dose of 10^5vg/cell.[71]

Immune Response to AAV

Originally thought to be minimally immunogenic, concerns regarding the innate and adaptive immune response to AAV have become more prominent as these vectors have expanded into clinical use. At this time, the innate response to AAV vectors appears low, especially compared to adenoviral vectors,[72] and seems to be mediated primarily through toll-like receptor (TLR) 9 signaling.[73] TLR9 may recognize viral genomes following uncoating in the cytoplasm, subsequently inducing pro-inflammatory cytokines and type I interferons (IFNs). The humoral response appears to be quite prevalent with an estimated 20-40% of humans having neutralizing antibody (NAb) titers > 1/20 against any given serotype.[74,75] Prevalence of NAbs depends on serotype; approximately 80% of humans have NAbs against AAV2.[74] Several animal and human clinical studies have shown that titers as low as 1:2 – 1:4 can prevent successful transduction and transgene expression.[67,76,77] In order for a humoral immune response to occur, B cells must recognize the viral capsid via membrane-bound immunoglobulins (B cell receptor), followed by internalization, processing and presentation by MHC II. Activated B cells can then either undergo clonal expansion and differentiation into antibody-secreting plasma cells or form germinal centers in the lymph nodes where memory B cells are formed.[78] In comparison to a seemingly consistent humoral response,

cell-mediated responses to AAV vectors appear to be less common.

A lack of significant CD4+ T cell responses may be because rAAVs do not readily transduce antigen-presenting cells (APCs).[79,80] Additionally, the weak activation of innate immunity by AAV limits the stimulation of maturation of APCs. Activated APCs are able to upregulate expression of MHC II and co-stimulatory molecules CD80/86 and CD40, as well as release pro-inflammatory cytokines and chemokines, thereby enhancing their ability to process and present antigen.[72] $CD8^+$ T cell responses to AAV have been shown in animal models and humans.[81,82] It is hypothesized that, following transduction and viral uncoating, some capsid remains in the cytosol and is processed by the proteasome prior to presentation on MHC I molecules. AAV capsid presentation by MHC I results in activation and expansion of capsid-specific $CD8^+$ memory T cells with subsequent removal of transduced cells. Cross-presentation by APCs is also possible with exogenous antigen taken up, processed and presented by MHC I to $CD8^+$ T cells instead of the normal MHC II pathway for such exogenous antigen.

Understanding the immune response to AAV is vital to its success as a gene therapy vector as the innate, humoral and cell-mediated immune response play major roles in vector effectiveness and host tolerance of AAV-mediated treatment modalities.

Conclusion

Trauma to the articular surface can precipitate irreversible loss of chondrocyte function and degradation of the ECM leading to OA. End-stage treatment for osteoarthritic joints in man is total joint replacement which can alleviate pain but is costly, often needs repeating, and does not offer return to full function. It is widely accepted that preventing the development of OA following articular cartilage injury would be much more efficient and useful. Quelling of the catabolic cascade and promotion of anabolism following injury may return physiologic balance to the cartilage and prevent development of OA and eventual loss of function. Gene therapy for cartilage repair targeting both axes of cartilage homeostasis is a promising avenue to achieve this goal.

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CHAPTER 2

IMPLANTATION OF rAAV5-IGF-I TRANSDUCED AUTOLOGOUS CHONDROCYTES IMPROVES CARTILAGE REPAIR IN FULL-THICKNESS DEFECTS IN THE EQUINE MODEL

Abstract

Cartilage injury often precipitates debilitating osteoarthritis due to poor healing capabilities. Autologous chondrocytes transduced with rAAV5-IGF-I were evaluated in chondral defects in a large animal model of cartilage repair. Cartilage was harvested from the talus of 24 horses; chondrocytes were isolated and stored frozen. Twenty million cells were cultured and transduced with 10^5 AAV vg/cell 48 hours prior to implantation. Chondrocytes from 8 horses were transduced with rAAV5-IGF-I, chondrocytes from 8 horses with rAAV5-GFP, and chondrocytes from 8 horses were not transduced. A 15mm full-thickness chondral defect was created arthroscopically in the lateral trochlear ridge of the femur in both femoropatellar joints. The treatment defect was filled with chondrocytes in fibrin. Control defects in the opposite limb received fibrin alone. rAAV5-IGF-I transduced chondrocytes resulted in significantly better healing scores at 8 week arthroscopy and 8 month necropsy when compared to the fibrin control. At 8 months, rAAV5-IGF-I defects had better histological scores than control and defects repaired with naïve chondrocytes. rAAV5-IGF-I defects had increased chondrocyte predominance and increased collagen type II, all features of hyaline-like repair tissue. The equine model closely approximates human cartilage healing; indicating AAV-mediated genetic modification of chondrocytes may be clinically beneficial to human patients.

Introduction

Damaged articular cartilage often precipitates painful and debilitating osteoarthritis (OA).[1] Joint injury and OA are often career-ending in athletes and are one of the most common causes of disability in the aging population. Cartilage has minimal to no intrinsic healing capabilities,[2] prompting investigation of varied methods to improve repair. Full-thickness lesions that are left untreated fill with fibrous tissue which is biochemically and biomechanically inferior to hyaline cartilage.[3] Techniques to improve cartilage repair have been investigated including marrow stimulation,[4] osteochondral transplantation (mosaicplasty),[5] and more recently autologous chondrocyte implantation (ACI).[6] Cell-based cartilage repair has shown promising long-term results in equine and human patients with production of hyaline-like repair tissue following transplantation of chondrocytes into chondral defects.[7] However, repair tissue may be further improved through the genetic manipulation of the donor chondrocytes.

Previous chondrocyte transplantation studies in the equine model have used allogeneic chondrocytes from juvenile donors[8] as these cells are easily accessible from donor animals, limit treatment to one surgical event and decrease donor site morbidity. However, the potential detrimental effect of the host immune response on healing continues to be of concern. Although chondrocytes are generally considered to be immunoprivileged when contained in the extra-cellular matrix (ECM),[9] they express MHC I and II[10] and could incite an immune response. Use of autologous chondrocytes eliminates potential immune responses to cells, which could benefit overall repair.

Insulin-like growth factor-I (IGF-I) has been identified as a critical anabolic and mitogenic protein in chondrocyte metabolism and cartilage repair.[11] IGF-I increases aggrecan and collagen type II content in chondrocyte cultures, including explants, monolayer and three-dimensional systems, as well as enhancing the repair potential of chondrocytes grafted into cartilage lesions in horses.[12,13] Additionally, it has been suggested that IGF-I plays a role in the healing of damaged cartilage as it partially protects and aids in the recovery of the ECM following experimentally induced damage with IL-I and TNF-α.[14,15] Although chondrocytes supplemented with exogenous IGF-I have been used in clinical practice,[16] the transitory nature of growth factor supplementation has led to investigation of gene therapy approaches.

Viral vectors including adenovirus and retrovirus have been examined for musculoskeletal gene therapy due to high transduction efficiencies but concerns regarding immunogenicity and genome integration have limited their use. Adeno-associated virus (AAV) may be a more feasible and clinically relevant vector as it lacks pathogenicity, can transduce dividing and non-dividing cells, has long-term transgene expression, and transduced cells appear to be minimally immunogenic.[17] Recombinant AAVs are not capable of productive infections and rarely integrate into host genomes. AAV, being a single-stranded DNA virus, requires second strand synthesis of the viral genome prior to transcription and expression of transgenes. More recently a self-complementary AAV (scAAV) has been developed to decrease the lag time between transduction and transgene expression as the genome can fold into dsDNA without the need for second strand synthesis.[18]

The objective of this study was to evaluate short- and long-term cartilage healing in full-thickness chondral defects repaired with autologous chondrocytes transduced *ex vivo* with a rAAV5 vector overexpressing IGF-I. We hypothesized that autologous chondrocytes overexpressing IGF-I would improve articular cartilage healing as measured by morphologic, histologic and biochemical parameters.

Materials and Methods

Adeno-associated vectors

Equine IGF-I cDNA was originally derived from RNA isolated from liver and cartilage from a 2 year old Thoroughbred horse. Three overlapping clones using primers derived from known sequence data for equine IGF-I (GenBank accession number U28070) were used to amplify the sequence. Full-length equine IGF-I cDNA and GFP were each subcloned into the rAAV transfer plasmid pHpa-trs-SK using SacII and Not sites. The transgenes were flanked by inverted terminal repeats and under control of the CMV promoter. scAAV5-IGF-I and scAAV5-GFP vectors were generated by the Research Vector Core at The Children's Hospital of Philadelphia (CHOP) in HEK293 cells using the triple plasmid transfection method in which cells were transfected with three different plasmids. The plasmids included: 1) rAAV-IGF-I or rAAV-GFP construct, 2) the AAV *rep* and *cap* genes and 3) the adenovirus helpervirus.

Cartilage biopsy and graft preparation

Twenty-four horses (11 geldings, 13 mares), between two and four years old (mean 2.9 years) and free of hindlimb musculoskeletal disease underwent cartilage biopsy using a protocol approved by the Institutional Animal Care and Use Committee. There were 13 Thoroughbreds, 6 Quarter Horses, and 5 American Paint Horses, with a mean (± SEM) weight of 460.2 (± 15.8) kg.

Horses were anesthetized and articular cartilage (2-3 g) was arthroscopically harvested from the non-weight bearing portions of the distal medial and lateral trochlear ridges of one randomly selected talus. Chondrocytes were isolated, expanded under monolayer culture conditions to 20 x 10^6 cells and then kept frozen in liquid nitrogen as previously described.[19] Chondrocytes were prepared for surgical implantation as previously described,[20] however, cells were transduced 48 hours prior to surgery in Opti-Mem (Invitrogen, Grand Island, NY) with rAAV5-IGF-I (*n*=8), rAAV5-GFP (*n*=8), or no virus as a negative control (*n*=8) at a dose of 10^5 vg/cell for 2 hours. Following transduction, chondrocytes were suspended in cryoprecipitated autogenous fibrinogen that was prepared from previously collected plasma.[21]

Chondrocyte implantation

Horses were anesthetized, synovial fluid was collected from both femoropatellar joints and bilateral arthroscopy was performed. A full-thickness chondral defect that extended down to, but not through, the subchondral plate, was created on the lateral trochlear ridge of

both femurs using a 15mm diameter fluted spade-bit cutter with a sharpened perimeter skirt (Special Devices, Grass Valley, CA) under arthroscopic guidance. Residual cartilage and calcified cartilage were removed using a loop curette. Each horse served as its own control with one defect being grafted with chondrocyte/fibrinogen mixture and the contralateral defect grafted with fibrin alone. Chondrocyte implantation was performed using helium gas for joint distension. A double-barreled syringe containing the fibrinogen/chondrocyte mixture in one syringe and calcium-activated bovine thrombin (500U/ml, Sigma, St Louis, MO) in the other syringe to polyermize the fibrinogen was used, providing an adhesive clot of chondrocytes. Once the clot became firm, the joint was lavaged with sterile fluid and put through repeated range of motion to visualize retention of the graft. Peri-operative pain management was achieved through use of epidural morphine and oral phenylbutazone administration. Horses were box stall rested for 4 weeks post-operatively, followed by 4 weeks of increasing amounts of walking.

Synovial fluid collection and analysis

Synovial fluid was collected from both femoropatellar joints immediately pre-operatively and then at days 4, 7, 14, 28, 56 and 224 post-implantation. Cytological analysis of the synovial fluid was performed with total and differential leukocyte count performed by Coulter (Beckman Coulter Inc., Fullerton, CA) and smear evaluation performed on Giesma-stained smears (LIDE Laboratories Inc., Florissant, MO). Synovial fluid was also analyzed for IGF-1 concentration using an ELISA (Quantikine, R&D Systems, Minneapolis, MN).

Arthroscopic evaluation at 8 weeks post-implantation

Eight weeks post-implantation surgery, arthroscopic second-look examination of defects in both joints was performed. The defects were examined, photographed and graded by two blinded observers (AJN,KO) using a semi-quantitative scoring system. Individual parameters included area covered with smooth white repair tissue, area of defect filled, amount of white tissue, graft-recipient tissue integration, and subchondral bone attachment. Following defect evaluation, a synovial biopsy was taken and fixed in 4% paraformaldehyde (PFA) for histology. Exercise was restricted to box stall rest for 2 weeks and then horses were allowed free pasture turnout for the remainder of the study period.

Gross evaluation and specimen collection

Eight months post-implantation horses were euthanized with a barbiturate overdose. The popliteal and inguinal lymph nodes were collected and fixed. The lateral trochlear ridges were exposed, photographed, and graded using the same semi-quantitative scoring system described above. An oscillating saw was used to remove a 5mm-wide rectangular osteochondral block containing the middle third of the repair tissue and extending 5mm beyond the proximal and distal edges of the graft. The blocks were fixed in 4% PFA and then decalcified in 10% ethylenediamine-tetra-acetic acid (EDTA) in preparation for histology and immunohistochemistry. Repair tissue from the medial third of the defect was harvested, along with a 5mm wide strip of perilesion cartilage and remote cartilage from the medial trochlear ridge. These tissues were snap frozen in liquid nitrogen and stored in -80^{o}C for later gene

expression and biochemical analysis. A 3 x 10mm biopsy of the synovial membrane and underlying joint capsule was collected from the cranial aspect of the femoropatellar joint and fixed in 4% PFA.

Morphologic analysis

Histology

Decalcified osteochondral blocks were embedded in paraffin, sectioned at 6μm, and stained with hematoxylin and eosin (H&E) to evaluate morphology, and toluidine blue to evaluate proteoglycan content and distribution in the extracellular matrix. Stained osteochondral blocks were graded by two blinded investigators (AJN, KO) using the semi-quantitative scoring system. Individual parameters included defect filling, chondrocyte predominance, perilesional cloning, subchondral bone attachment, tissue integration, surface fibrillation, tidemark reformation, toluidine blue staining, and collagen type II predominance. Synovial membrane was sectioned and stained with H&E for scoring. Popliteal and ileofemoral lymph nodes collected from both limbs were also stained with H&E and scored by a veterinary pathologist. The B-cell domain, T-cell domain, medullary cords and medullary sinuses were evaluated for inflammatory reaction.

Collagen immunohistochemistry

Osteochondral sections were deparaffinized, rehydrated, and treated with 5μg/mL

hyaluronidase (Sigma Chemical Co.) at 37^{o}C for 60 min prior to blocking with normal goat serum. Sections were then incubated with polyclonal rat anti-bovine type II collagen primary antibody (1:100) (courtesy of Dr. Michael Cremer, VA Hospital, Memphis, TN) or polyclonal rabbit anti-equine type I collagen primary antibody. A secondary biotinylated goat- anti-rat or anti-rabbit antibody (ABC Staining System, Santa Cruz Biotechnology, Dallas, TX) was applied to sections, followed by streptavidin conjugated peroxidase to catalyze chromagen development in 3,3'-diaminobenzidine tetrachloride (Sigma-Aldrich, St Louis, MO). Sections were counterstained with hematoxylin and scored based on amount and intensity of staining.

Biochemical analyses

Frozen lesion, perilesion and remote tissues were pulverized in liquid nitrogen in a freezer mill (6750 Freezer Mill, Spex Certiprep, Metuchen, NJ), lyophilized, and used for glycosaminoglycan (GAG), DNA, and type II collagen assays. The dimethylmethylene blue (DMMB) spectrophotometric assay was used to estimate proteoglycan content in tissues. Chondroitin-4 sulfate was used to establish a standard curve and the OD determined at 525nm.[22] Total DNA content was determined from papain digested tissues that were incubated for 24h at 65^{o}C and then mixed with bisbenzimide compound (compound 33258, Hoechst, Sigma-Aldrich, St Louis, MO) for quantification by fluorometric assay, using an excitation wavelength of 348 nm and an emission wavelength of 456 nm. Calf thymus DNA was used to establish a standard curve. Collagen type II content was assessed using a multispecies collagen type II ELISA (Chondrex Inc., Redmond, WA). Lypophilized cartilage was pretreated and digested with guanidine, pepsin and elastase prior to assay by ELISA. The

OD values were read at 490 nm.

Gene Expression analysis

Following pulverization in a freezer mill, tissues were mechanically homogenized and RNA was isolated from tissues using the PerfectPure RNA Tissue Kit (5 Prime, Gaithersburg, MD). Purity and concentration of the RNA was assessed by UV microspectrophotometry (NanoDrop 2000 Spectrophotometer, Thermo Scientific, Waltham, MA). Gene expression was quantified by real-time PCR using the Taqman One-Step RT-PCR technique (Absolute Quantitative PCR; ABI PRISM 7900 HT Sequence Detection System, Applied Biosystems, Foster City, CA). Gene expression of IGF-I, collagen type II (col II), aggrecan (agg), and collagen type I (col I) was measured in lesion, perilesion and remote tissue. All samples were run in duplicate. Primer Express Software Version 2.0 (Applied Biosystems, Foster City, CA) was used to design equine primers and dual-labeled fluorescent probes [6-carboxyfluorescein (FAM) as the 5' label (reporter dye) and 5-carboxymethylrhodamine (TAMRA) as the 3' label (quenching dye)]. Sequences for primers and probes were generated from GenBank (National Institutes of Health, Bethesda, MD) or available from clones with the laboratory of Dr. Alan Nixon. Primer sequences were as follows: forward, 5'-CGGCTTTGGTGACTCTAGATAACC-3' and reverse, 5'-CCATGGTAGGCACAGC GACTA-3' for 18S; forward, 5'-TGTACTGCGCACCCCTCAA-3' and reverse, 5'-TTGT GTTCTTCAAATGTACTTCCTTCTG-3' for IGF-I; forward, 5'-CGCTGTCCTTCGGTGT CA-3' and reverse, 5'-CTTGATGTCTCCAGGTTCTCCTT-3' for coll II; forward, 5'-GATGCCAC TGCCACAAAACA-3' and reverse, 5'-GATGCCACTGCC ACAAAACA-3'

for aggrecan; forward, 5'-GTACCACGACCGAGCCGTAT-3' and reverse, 5'-GATCACGT CATCGCACAACAC-3' for col I. The total copy number of mRNA was determined using a validated standard curve and these values were normalized to the housekeeping gene 18S.

Statistical analysis

Each horse in the study provided multiple observations; one chondral defect filled with either 1) rAAV5-IGF-I chondrocytes 2) rAAV5-GFP chondrocytes or 3) naïve chondrocytes, and the contralateral defect filled with fibrin vehicle. Several of the outcome parameters (e.g. synovial fluid IGF-I concentration) were also repeatedly measured over time. In order to account for possible correlation between observations made in the same horse a mixed effects model, with horse as a random effect, was used for analysis. Day was treated as a categorical variable. Histograms were visually inspected for a Gaussian distribution and Shapiro-Wilk test was performed. Multiple comparisons for differences in parameters for each interaction were made with a Tukey's *post hoc* test or Bonferroni correction. Specific linear contrasts were fitted to the model where appropriate to examine the nature of the interactions. Statistical analysis was performed using JMP. The level of significance was set at $p<0.05$.

Results

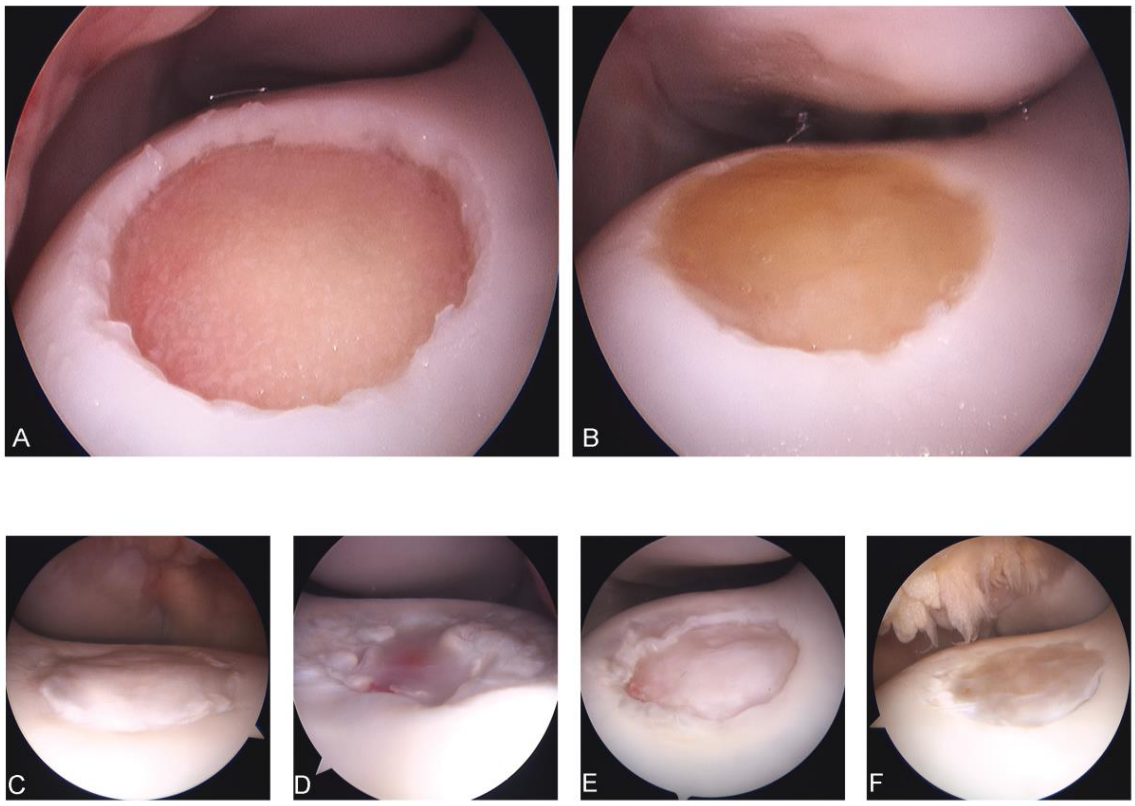
Arthroscopic cartilage harvest yielded autologous chondrocytes for implantation into chondral defects

Arthroscopic cartilage harvest of the non-weight bearing regions of the distal medial and lateral trochlear ridges of the talus was achieved in each horse (n=24) yielding 1.5-2.5g of cartilage. Collagenase digestion of cartilage produced approximately 15 x 10^6 chondrocytes that were expanded in culture to a total of 20 x 10^6 cells prior to implantation. Full-thickness chondral defects were created successfully in the lateral trochlear ridge of both femurs using an arthroscopically guided 15mm diameter fluted spade-bit cutter (**Figure 2.1a**). Calcified cartilage was completely debrided from the surface of the defect using a curette. Horses were randomly assigned to a treatment group in which one chondral defect was filled with 1) fibrin containing chondrocytes transduced with rAAV5- IGF-I (*n*=8), 2) fibrin containing chondrocytes transduced with rAAV5-GFP (positive control) (*n*=8) or 3) fibrin containing naïve, untransduced chondrocytes (*n*=8). The contralateral joint of each horse was grafted with the autogenous fibrin vehicle alone (*n*=24). All grafts stabilized within several minutes and remained stable while joints were moved through range of motion (**Figure 2.1b**).

Prior to implantation of autologous chondrocytes transduced *ex vivo* with the rAAV5 vector, successful rAAV5-mediated IGF-I expression by equine chondrocytes *in vitro* was demonstrated in our lab.[23] At 14 days post-transduction, expression of IGF-I in chondrocytes transduced with 1 x 10^5 vg/cell was 115-fold higher than expression of IGF-I in untransduced

cells as quantified by qPCR. IGF-I concentration in the supernatants of rAAV5-IGF-I transduced chondrocytes was also significantly increased at 14 days post-transduction (55.7±8.5ng/ml) over untransduced cells (0.24±0.1ng/ml).

Figure 2.1. Arthroscopic images of empty (a), implanted (b) and healing defects 8 weeks post-implantation (c-f) with associated healing scores (g). (a) A full-thickness, 15mm diameter chondral defect in the lateral trochlear ridge of the femur prior to implantation. (b) The same defect after chondrocyte implantation. The implant has been stabilized by polymerization of the fibrin vehicle. (c) Defect repaired with rAAV5-IGF-I transduced chondrocytes showing good defect fill and white "cartilage-like" tissue compared to the defects filled with (d) rAAV5-GFP transduced chondrocytes, (e) naïve chondrocytes or (f) fibrin alone. Independent parameter scores and total scores for defect healing at 8 weeks are shown (g) in which lower scores represent superior healing and higher scores indicate more abnormal tissue. Data in the table represented as mean ± SEM. Different letters denote significant differences between groups. $p<0.05$



G

Treatment Group	Area Covered with Smooth Repair Tissue	Area of Defect Filled	Tissue Color	Graft-recipient Tissue Integration	Subchondral Bone Attachment	Total Score
rAAV5-IGF-I	1.58±0.32[a]	1.36±0.35	1.51±0.32[a]	0.73±0.32	0.35±0.42	5.35±1.30[a]
rAAV5-GFP	3.10±0.32[b]	2.51±0.35	2.87±0.32[b]	1.54±0.32	0.93±0.42	11.05±1.30[b]
Naïve Chondrocyte	2.32±0.32[ab]	2.25±0.35	2.62±0.32[ab]	0.85±0.32	0.46±0.42	8.56±1.30[ab]
Control	3.12±0.18[b]	2.38±0.20	2.96±0.18[b]	1.08±0.20	1.25±0.24	10.79±0.73[b]

Chondral defects repaired with rAAV5-IGF-I transduced chondrocytes had improved healing at 8 weeks post-implantation

Short-term healing of the defects was assessed arthroscopically at 8 weeks post-implantation at which time all defects were partially filled with repair tissue. Representative images from second-look arthroscopy from all 4 treatment groups are shown (**Figure 2.1c-f**). Healing was evaluated using a scoring system based on five parameters (area covered with smooth white repair tissue, area of defect filled, amount of white tissue, graft-recipient tissue integration, and subchondral bone attachment) yielding independent scores for each category and a composite score. Healing scores closest to zero reflected repair tissue most similar to native cartilage, while higher scores reflected the most abnormal repair tissue. Defects repaired with rAAV5-IGF-I chondrocytes had significantly more area covered with smooth repair tissue (**Figure 2.1c**) than defects repaired with rAAV5-GFP chondrocytes (**Figure 2.1d**) or fibrin alone (**Figure 2.1f**) and this was reflected by rAAV5-IGF-I defects having significantly lower (better) healing scores (**Figure 2.1g**). Additionally, rAAV5-IGF-I treated defects had significantly more area covered with white repair tissue (**Figure 2.1c**) than defects repaired with rAAV5-GFP chondrocytes (**Figure 2.1d**) or fibrin alone (**Figure 2.1f**). Overall, defects repaired with rAAV5-IGF-I had the lowest (best) total arthroscopic healing score (mean±SEM; 5.35±1.30) compared to defects filled with rAAV5-GFP (11.05±1.30), naïve chondrocytes (8.56±1.30) and fibrin alone (10.79±0.73) (**Figure 2.1g**). The differences between rAAV5-IGF-I and rAAV5-GFP or fibrin alone were significant.

rAAV5-IGF-I transduced chondrocytes increase synovial fluid IGF-I concentration without causing significant inflammatory changes

In order to assess transgene expression following chondrocyte implantation, IGF-I content in the synovial fluid of femoropatellar joints was analyzed over time using a mixed effects model to account for repeated measures. The effect of day (F ratio = 90.6; $p<0.0001$) and treatment group (F ratio = 3.8; $p<0.012$) were both significant, as was the interaction between day and treatment group (F ratio = 2.2; $p<0.004$). IGF-I content in the synovial fluid increased in all treatment groups immediately post-operatively with return to baseline by day 56 (**Figure 2.2**). Synovial fluid IGF-I concentrations obtained from femoropatellar joints treated with rAAV5-IGF-I chondrocytes was significantly increased over joints treated with rAAV5-GFP chondrocytes (Bonferroni adjusted $p=0.025$, linear contrast) and fibrin alone (Bonferroni adjusted $p<0.0001$, linear contrast) at day 4 (**Figure 2.2**). At day 7 post-operatively, IGF-I concentrations obtained from joints treated with rAAV5-IGF-I chondrocytes were still significantly increased over joints treated with rAAV5-GFP chondrocytes (Bonferroni adjusted $p=0.014$, linear contrast) and fibrin alone (Bonferroni adjusted $p=0.0006$, linear contrast) (**Figure 2.2**). IGF-I levels were highest at 4 days post-operatively with rAAV5-IGF-I treated joints having 202.4 ng/ml (± 24.4) compared to 145.3 ng/ml (±17.7) in rAAV5-GFP joints, 172.2 ng/ml (±85.4) in naïve chondrocyte joints and 125.7 ng/ml (±12.9) in fibrin joints (**Figure 2.2**).

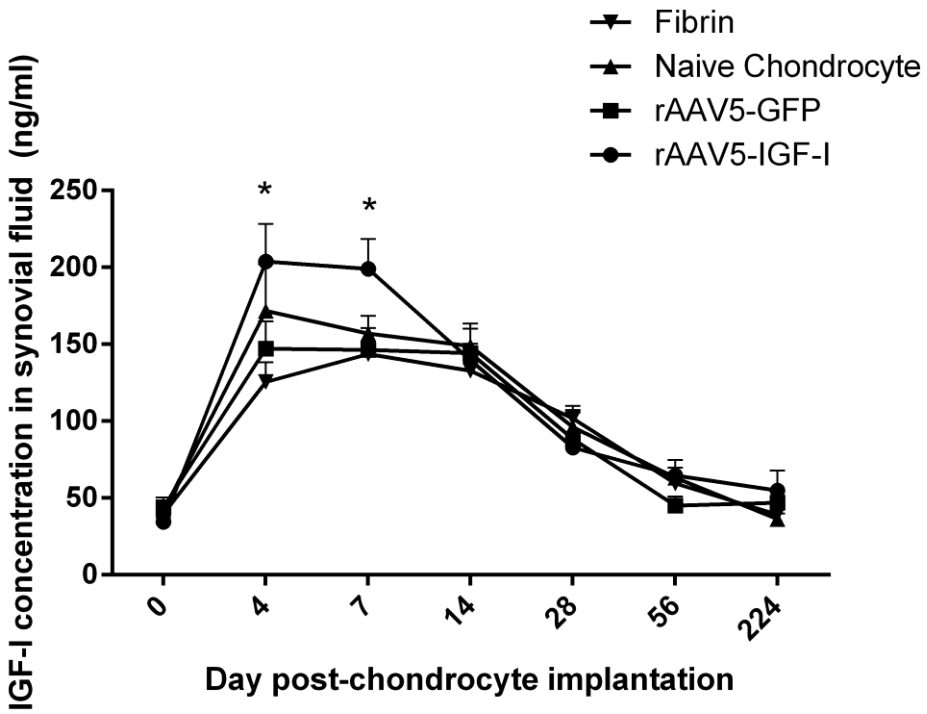
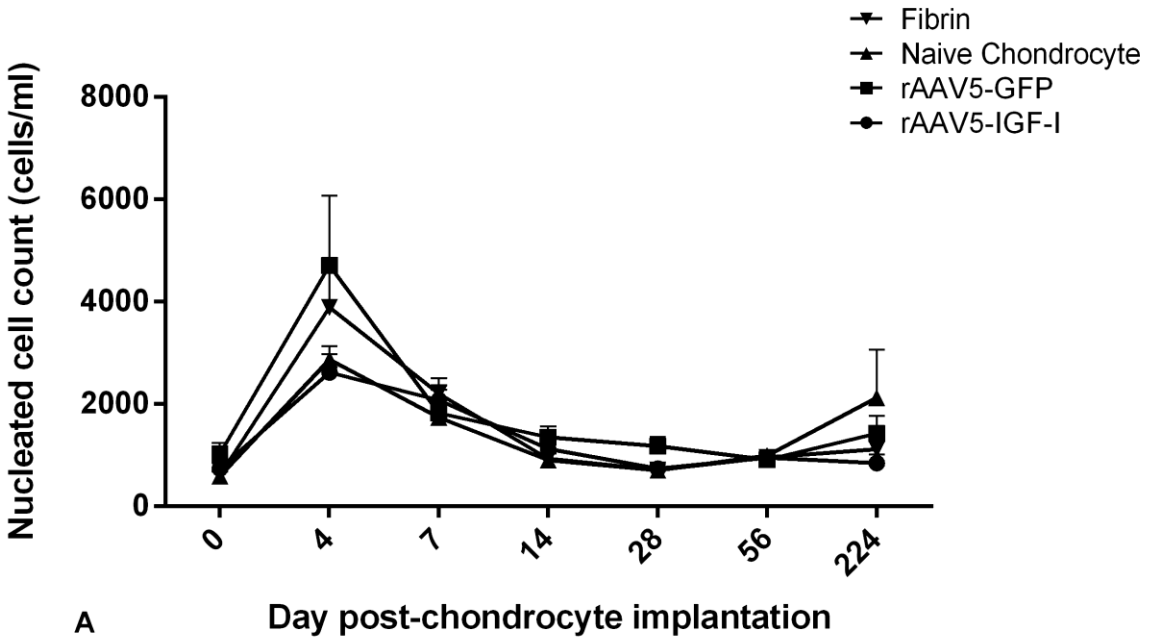


Figure 2.2. Concentrations (means ± SEM) of IGF-I in the femoropatellar synovial fluid over time from joints treated with rAAV5-IGF-I transduced chondrocytes, rAAV5-GFP transduced chondrocytes, naïve chondrocytes, or fibrin alone. * Significant difference between rAAV5-IGF-I treated joints compared to rAAV5-GFP and fibrin treated joints. $p<0.05$

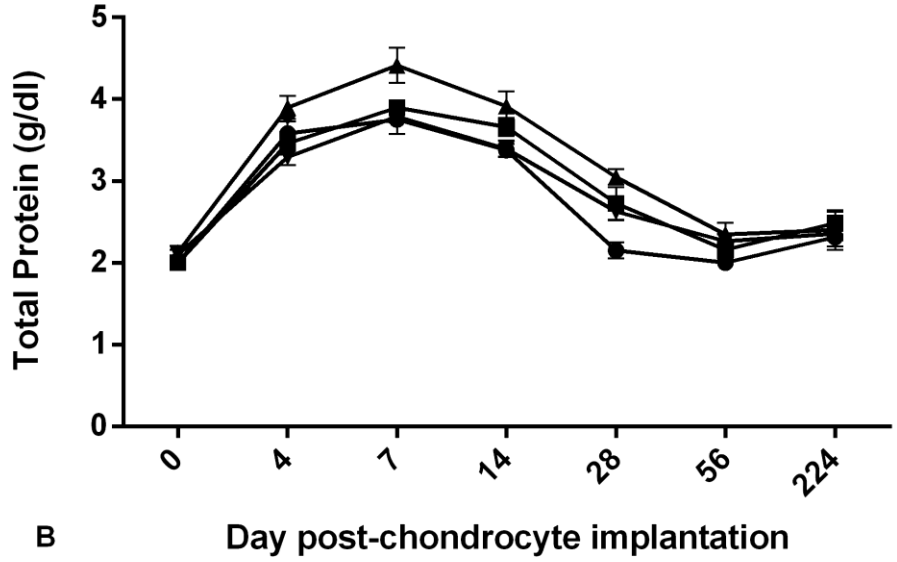
The correlation between IGF-I concentrations in femoropatellar synovial fluid and healing of defects was also evaluated. There was a weak, but significant ($p<0.05$), correlation between peak IGF-I concentrations at day 4 and 7 with 8 week arthroscopic healing scores, 8 month gross healing scores and total histological scores. Horses with higher IGF-I concentrations post-implantation had better healing in the short- and long-term.

Further analysis of synovial fluid composition was performed to assess the inflammatory response of the joint to the vectors. The nucleated cell count (NCC) increased over baseline in femoropatellar joints of all treatment groups 4 days post-operatively, however, these increases were only significantly different compared to preoperative NCCs in horses treated with rAAV5-GFP chondrocytes (4712.5±1366.8cells/μL) and fibrin alone (3890.5±709.8 cells/μL) (**Figure 2.3**). Horses treated with rAAV5-GFP chondrocytes had a significantly higher NCC 4 days post-operatively compared to rAAV5-IGF-I (2616.7±360.0cells/μL; Bonferroni adjusted $p=0.0042$, linear contrast) and naïve chondrocytes (2871.4±260.7cells/μL; Bonferroni adjusted $p=0.015$, linear contrast) (**Figure 2.3a**). All NCCs decreased by day 7 postoperatively with no significant differences between pre- and postoperative counts. Synovial total protein (TP) levels increased significantly over baseline in all femoropatellar joints immediately following surgery with no significant differences between treatment groups (**Figure 2.3b**). Total protein concentrations remained significantly elevated above preoperative levels until day 56 postoperatively in all groups except rAAV5-IGF-I treated joints in which total protein concentrations returned to baseline by day 28 (**Figure 2.3b**).

Figure 2.3. Synovial fluid composition following chondrocyte implantation including (a) nucleated cell count (NCC) and (b) total protein (g/dl). No significant differences were noted between groups.


Fibrin
Naive Chondrocyte
rAAV5-GFP
rAAV5-IGF-I
Nucleated cell count (cells/ml)
8000
6000
4000
2000
0
0
4
7
14
28
56
224
Day post-chondrocyte implantation

A

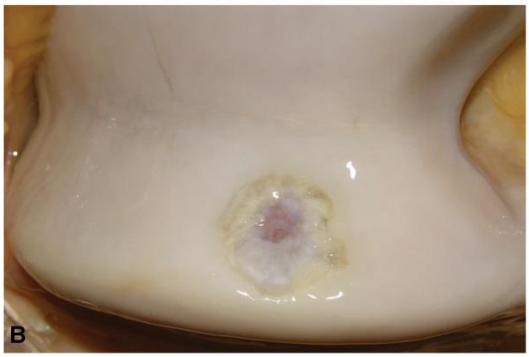

Total Protein (g/dl)
5
4
3
2
1
0
0
4
7
14
28
56
224
Day post-chondrocyte implantation

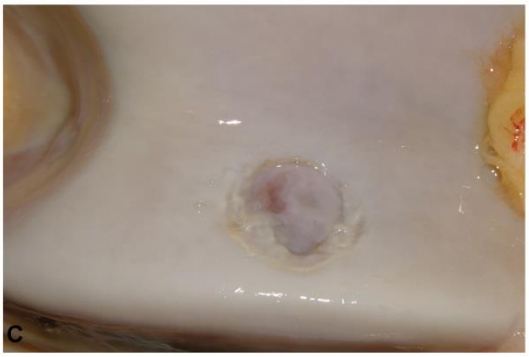
B

rAAV5-IGF-I transduced chondrocytes led to improved long-term healing with increased collagen type II content

Long-term healing of defects was assessed at 8 months post-implantation by means of gross and histologic healing scores. Gross images of healing defects from the 4 treatment groups (**Figure 2.4a-d**) revealed improved healing of rAAV5-IGF-I treated defects. Individual parameter and total gross scores presented in **Figure 2.4e** show defects repaired with rAAV5-IGF-I chondrocytes had significantly lower (better) total healing scores (mean±SEM; 4.13±0.69) than defects repaired with naïve chondrocytes (6.99 ±0.69) and fibrin alone (6.75 ±0.39). rAAV5-IGF-I treated defects had lower total healing scores than defects treated with rAAV5-GFP chondrocytes (5.75; SE±0.69) but this difference did not reach significance. Improved gross healing in rAAV5-IGF-I defects was reflected in the histological healing observed in osteochondral sections stained with hematoxylin & eosin. Representative images of all 4 treatment groups are shown (**Figure 2.5a-d).** Histological scoring at 8 months post-implantation revealed significantly improved (lower) total healing in rAAV5-IGF-I treated defects (mean±SEM; 12.47±1.26) compared to defects treated with naïve chondrocytes (18.32±1.26) or fibrin alone (18.58±0.77) (**Table 2.1**). Although total healing scores from defects repaired with rAAV5-IGF-I (12.47±1.26) were better than those repaired with rAAV5-GFP (15.97±1.26), these differences were not significant (**Table 2.1**).

Figure 2.4. Gross appearance of healing defects 8 months post-implantation (a-d) with associated gross healing scores (e). The gross images (a-d) and arthroscopic images in Fig. 2a-d are taken from the same defects. (a) Gross appearance of a defect repaired with rAAV5-IGF-I transduced chondrocytes compared to, (b) a defect repaired with rAAV5-GFP, (c) a defect repaired with naïve chondrocytes, and (d) a defect repaired with fibrin alone. Independent parameter scores and total scores for defect healing at 8 months are shown (e) in which lower scores represent superior healing and higher scores indicate more abnormal tissue. Data in the table represented as mean ± SEM. Different letters denote significant differences between groups. $p<0.05$





Treatment Group	Area Covered with Smooth Repair Tissue	Area of Defect Filled	Tissue Color	Graft-recipient Tissue Integration	Subchondral Bone Attachment	Total Score
rAAV5-IGF-I	1.13±0.24[a]	0.87±0.23	1.13±0.26	0.81±0.27[a]	0.25±0.27	4.13±0.69[a]
rAAV5-GFP	1.63±0.24[ab]	1.53±0.23	1.25±0.26	0.89±0.27[a]	0.53±0.27	5.75±0.69[ab]
Naïve Chondrocyte	2.13±0.24[b]	1.60±0.23	1.75±0.25	0.96±0.27[ab]	0.48±0.27	6.99±0.69[b]
Control	1.50±0.14[ab]	1.29±0.13	1.54±0.14	1.67±0.16[b]	0.96±0.27	6.75±0.39[b]

E

Figure 2.5. Photomicrographs of osteochondral sections taken from repair tissue at 8 months post-implantation and stained with hematoxylin and eosin. (a) rAAV5-IGF-I transduced chondrocytes show more chondrocyte predominance, improved tissue organization and less fibrous tissue than defects treated with either (b) rAAV5-GFP transduced chondrocytes, (c) naïve chondrocytes, or (d) fibrin alone. Inset boxes are enlarged to show tissue architecture and attachment at the subchondral bone interface. (Bar = 5mm; Inset bar = 500μm)

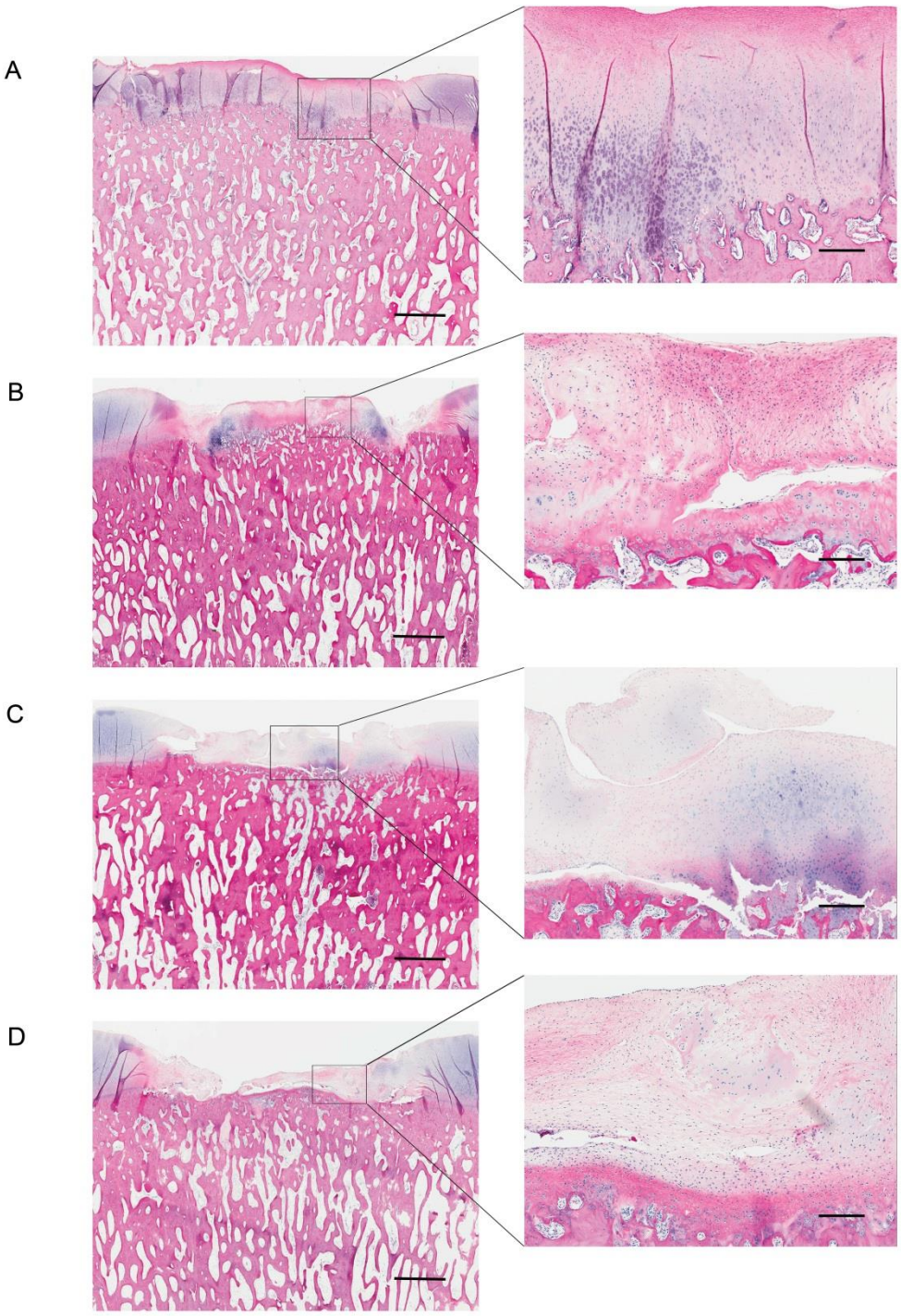

A
B
C
D

Table 2.1. Histologic healing scores of lesions at 8 months post-implantation

Treatment Group	Defect Filling (%)	Chond Predom	Perilesion Cloning	Subchond Bone Attach	Perimeter Attach	Surface Fibri-llation	Tidemark	Tol Blue Staining %	Coll type II IHC	Total Score
rAAV5-IGF-I	0.92±0.42	1.76±0.22[a]	0.35±0.2[a]	0.65±0.47	0.57±0.22	0.58±0.29	2.25±0.17[a]	3.23±0.2 [a]	2.09±0.33[a]	12.47±1.26[a]
rAAV5-GFP	1.53±0.42	2.38±0.22[ab]	0.5± 0.22[a]	1.30±0.47	0.82±0.22	1.02±0.29	2.25±0.17[a]	3.50±0.2 [ab]	2.63±0.33[ab]	15.97±1.26[ab]
Naïve Chond	1.55±0.42	2.49±0.22[ab]	0.78±0.22[ab]	1.30±0.47	1.23±0.22	1.52±0.29	2.63±0.17[ab]	3.90±0.2 [ab]	3.02±0.33[ab]	18.32±1.26[b]
Fibrin	1.25±0.25	2.71± 0.12[b]	1.25±0.12[b]	1.08±0.27	1.08±0.13	1.29±0.18	2.88±0.9[b]	3.88±0.11 [b]	3.17±0.19[b]	18.58±0.77[b]

Data presented as mean ± SEM. Results analyzed using a mixed effects model with horse as a random effect. Different letters denote significant differences between groups. $p<0.05$

Proteoglycan content (μg glycosaminoglycan/mg cartilage) of repair tissue at 8 months post-implantation was significantly higher in rAAV5-IGF-I treated defects (mean±SEM; 24.5±2.32μg/mg) compared to rAAV5-GFP treated defects (14.5±2.48μg/mg), however, there were no significant differences in proteoglycan between rAAV5-IGF-I, naïve chondrocytes (19.0±2.30μg/mg) and fibrin treated defects (23.3±1.43μg/mg) (**Table 2.2**). Interestingly, rAAV5-IGF-I had the most toluidine blue staining on histological samples, although these differences were only significant between rAAV5-IGF-I treated defects and fibrin treated defects (**Table 2.1**). Across all 4 treatment groups, proteoglycan content in repair tissue (20.3±2.46μg/mg) was approximately 1/3 the amount of proteoglycan content present in perilesion (67.4±2.53μg/mg) and remote cartilage (63.9±1.63μg/mg) supportive of moderate regeneration of the ECM at 8 months. There were no differences in DNA content of repair tissue across the 4 treatment groups (**Table 2.2**). Repair tissue DNA content was less than that of perilesion or remote tissues in all 4 groups indicating that repair tissue had decreased cellularity compared to native cartilage.

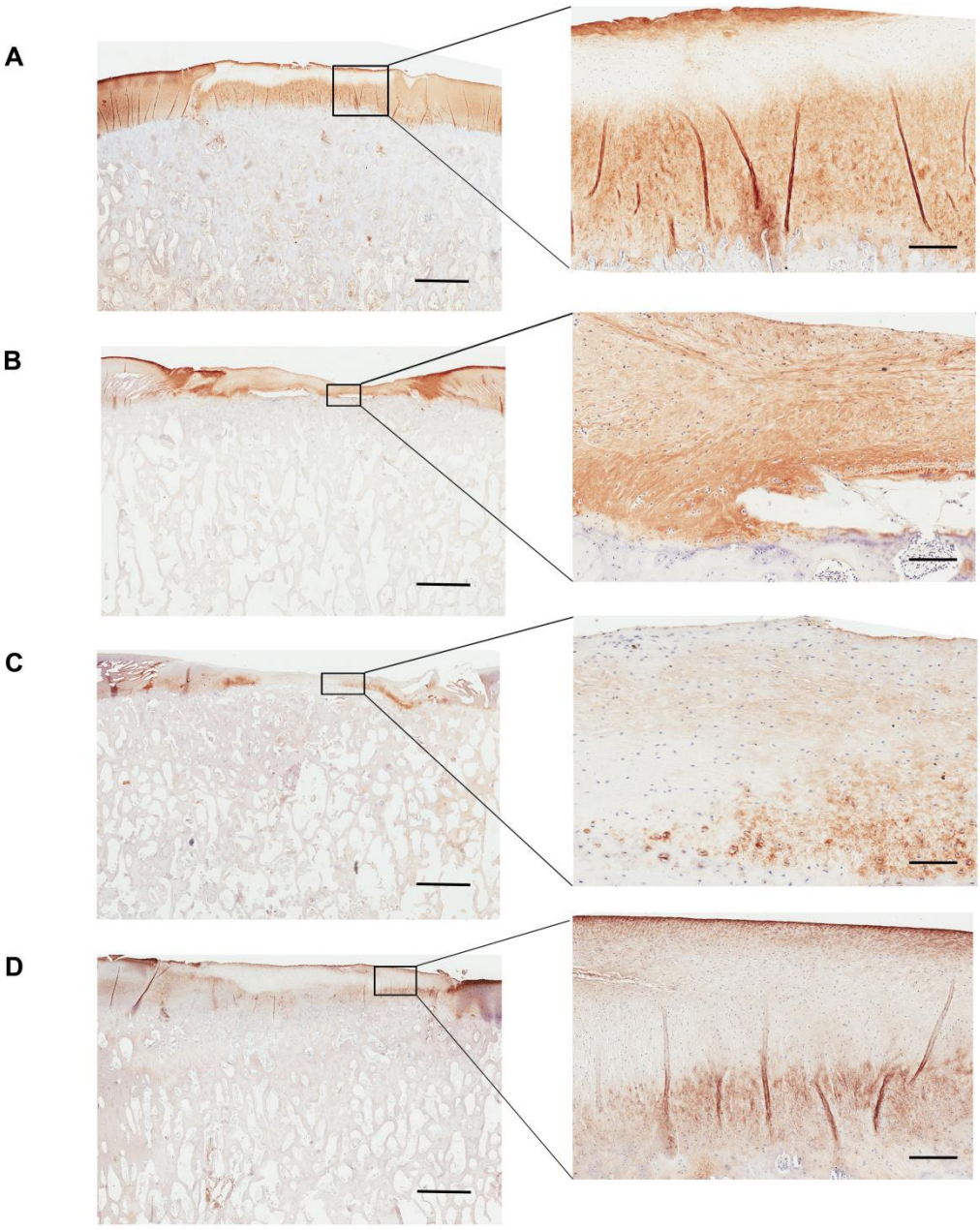
Collagen type II immunoreaction was more extensive in rAAV5-IGF-I treated defects compared to the other treatment groups (**Figure 2.6a-d**). Immunohistochemistry scoring of histological samples supported this finding with more collagen type II content in rAAV5-IGF-I treated defects, although these differences were only significant when compared to fibrin treated defects (**Table 2.1**). Increased collagen type II content in rAAV5-IGF-I treated defects was confirmed by ELISA, with significantly more collagen type II in rAAV5-IGF-I treated defects compared to defects treated with rAAV5-GFP chondrocytes, naïve chondrocytes or fibrin alone (**Figure 2.7**).

Table 2.2. Biochemical analysis of lesion cartilage 8 months post-implantation.

Treatment Group	Glycosaminoglycan	DNA
rAAV5-IGF-I	24.5 (2.32)[a]	7.34 (1.30)
rAAV5-GFP	14.5 (2.48)[b]	4.34 (1.14)
Naïve Chondrocytes	19.0 (2.30)[ab]	5.82 (1.30)
Fibrin	23.3 (1.43)[ab]	7.32 (0.71)

Data presented as mean ± SEM. Results analyzed using a mixed effects model with horse as a random effect. Different letters denote significant differences between groups. $p<0.05$

Figure 2.6. Collagen type II formation in repair tissue at 8 months post-implantation. (a) rAAV5-IGF-I transduced chondrocytes show increased collagen type II (brown staining) compared to defects repaired with (b) rAAV5-GFP transduced chondrocytes, (c) naïve chondrocytes, or (d) fibrin alone. (Bar = 5mm; Inset bar = 500μm)


A
B
C
D

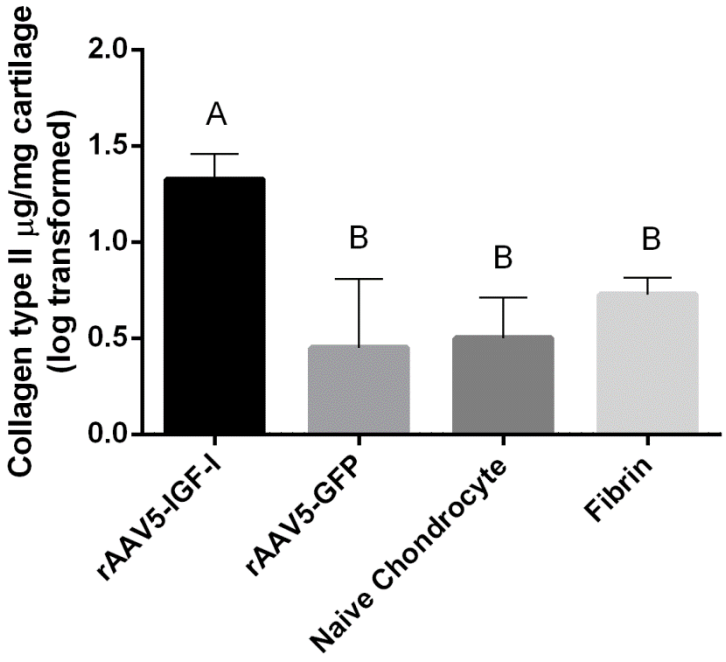


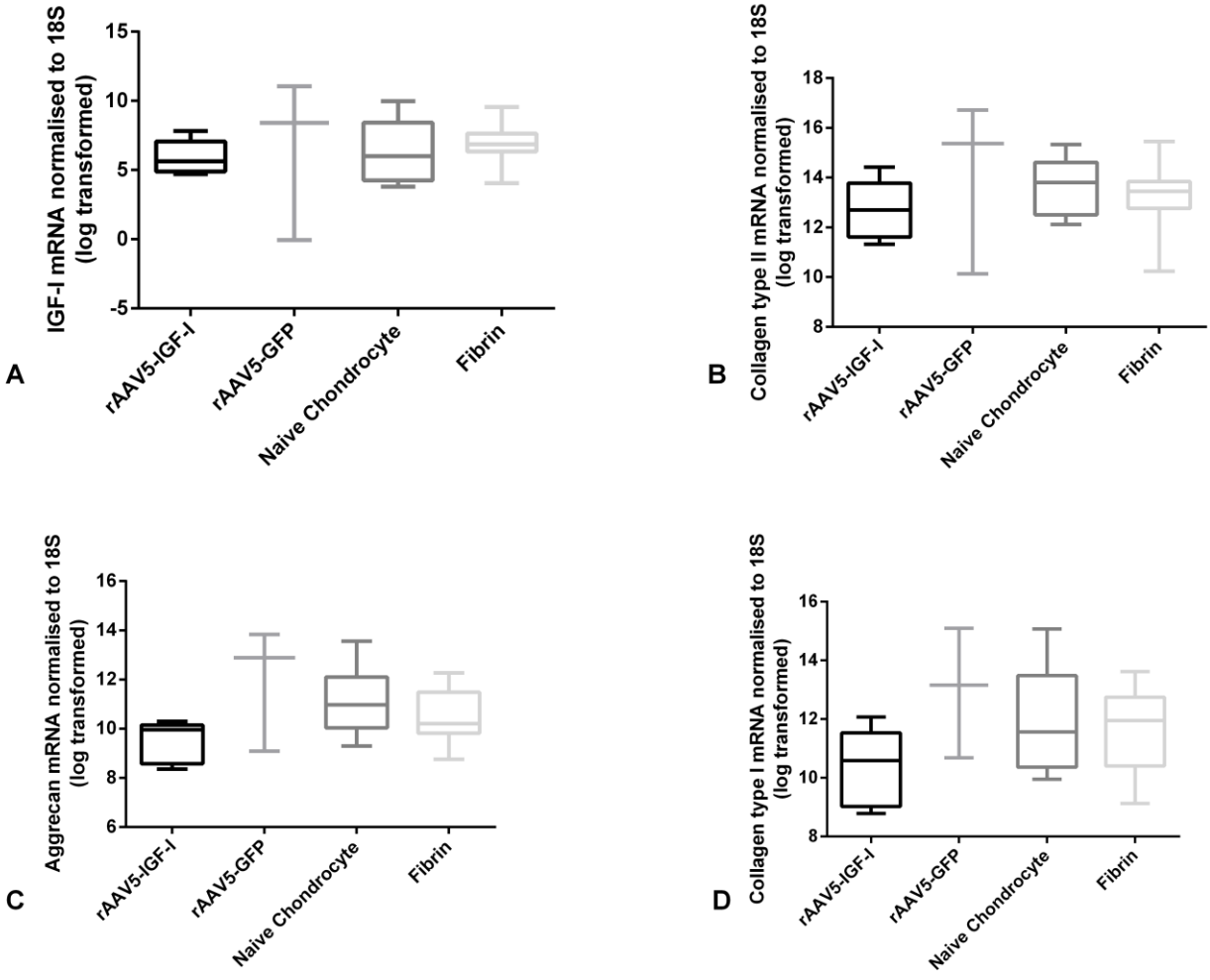
Figure 2.7. Log transformed (± SEM) collagen type II content in repair tissue in rAAV5-IGF-I, rAAV5-GFP, naïve chondrocyte and fibrin treated (control) defects. Tukey's classification letters indicate significant differences between treatment groups.

Expression of genes associated with hyaline cartilage including IGF-I, aggrecan, collagen type II and collagen type I were assessed in repair tissue, perilesion tissue and remote tissue by qRT-PCR. At 8 months post-implantation, there were no differences in expression of IGF-I, aggrecan, collagen type II or collagen type I in repair tissue from defects repaired with rAAV5-IGF-I chondrocytes, rAAV5-GFP chondrocytes, naïve chondrocytes or fibrin alone (**Figure 2.8a-d**). No differences in gene expression were noted in the perilesion or remote tissue from between any of the treatment groups.

Implantation of chondrocytes transduced *ex vivo* with a rAAV5 vector did not cause a significant inflammatory response in synovium

Synovial biopsies were collected during arthroscopic examination at 8 weeks post-implantation and at 8 months post-implantation. Synovium was evaluated and scored for changes in villus architecture, subintimal fibrosis, intimal thickening, increased vasculature, or inflammatory cell infiltration indicative of an inflammatory response. Synovial biopsies collected at 8 weeks and 8 months post-implantation showed no significant differences between treatment groups. Treatment with rAAV5 did not appear to cause a significant inflammatory reaction in the synovial tissue of the femoropatellar joint consistent with the synovial fluid profiles observed between the groups. Biopsies of the ileofemoral and popliteal lymph nodes (main draining lymph nodes of the femoropatellar joints) taken at necropsy showed significantly more reaction in ileofemoral than popliteal lymph nodes within in all treatment groups, however, no differences were noted in popliteal or ileofemoral lymph node reactivity between any of the treatment groups.

Figure 2.8. Quantitative polymerase chain reaction (qPCR) data showing expression of (a) IGF-I, (b) collagen type II, (c) aggrecan, and (d) collagen type I in repair tissue from defects treated with rAAV5-IGF-I chondrocytes, rAAV5-GFP chondrocytes, naïve chondrocytes or fibrin alone (mean; min-max). No significant differences were noted between the 4 treatment groups (18S RNA, 18S ribosomal ribonucleic acid).


A
IGF-I mRNA normalised to 18S (log transformed)
15
10
5
0
-5
rAAV5-IGF-I
rAAV5-GFP
Naive Chondrocyte
Fibrin
B
Collagen type II mRNA normalised to 18S (log transformed)
18
16
14
12
10
8
rAAV5-IGF-I
rAAV5-GFP
Naive Chondrocyte
Fibrin
C
Aggrecan mRNA normalised to 18S (log transformed)
16
14
12
10
8
6
rAAV5-IGF-I
rAAV5-GFP
Naive Chondrocyte
Fibrin
D
Collagen type I mRNA normalised to 18S (log transformed)
16
14
12
10
8
rAAV5-IGF-I
rAAV5-GFP
Naive Chondrocyte
Fibrin

Discussion

Implantation of autologous chondrocytes transduced with rAAV5-IGF-I *ex vivo* resulted in improved repair of full-thickness chondral defects of the lateral trochlear ridge of the horse, both in the short-term (8 weeks post-implantation), and long-term (8 months post-implantation). These grafted defects showed features of hyaline-like repair tissue including hyaline-like tissue architecture, chondrocyte predominance and collagen type II abundance. The total histologic scores of rAAV5-IGF-I treated defects were significantly improved over defects treated with naïve chondrocytes and fibrin alone. Collagen type II immunohistochemistry scores were also significantly improved in rAAV5-IGF-I treated defects compared to defects treated with fibrin alone. These results were further validated by collagen type II ELISA that showed significantly increased collagen type II protein in rAAV5-IGF-I treated defects over defects treated with rAAV5-GFP chondrocytes, naïve chondrocytes or fibrin alone. Upregulation of collagen type II in chondrocytes with IGF-I supplementation has been shown previously.[24,25] The increased collagen type II levels in our study are likely due to overexpression of IGF-I by the rAAV-IGF-I vector.

IGF-I has been shown to play a major role in articular cartilage homeostasis and anabolism.[14,26] The addition of exogenous IGF-I to chondrocyte grafts has been shown to improve repair of chondral defects *in vivo,* however, incomplete chondrogenesis was noted at 8 months.[12] We sought to increase longevity of IGF-I supplementation in repair tissue through use of a rAAV5 vector. Significantly increased levels of IGF-I protein were noted in the synovial fluid of rAAV5-IGF-I treated joints at 4 and 7 days post-implantation

suggestive of transgene expression. Interestingly, there was a significant correlation between IGF-I concentrations at days 4 and 7, and healing at 8 weeks and 8 months. Horses with higher IGF-I concentrations post-implantation were more likely to have superior healing. The decrease in synovial fluid IGF-I concentration after day 7 was not unexpected as the femoropatellar joint is a voluminous joint and the chondral defect containing transduced cells is relatively small. The inevitable dilution of IGF-I produced by transduced cells would make increases in IGF-I protein difficult to detect unless they were quite large. Expression profiles from chondrocytes transduced *in vitro* with rAAV5-IGF-I showed significantly elevated IGF-I concentrations up to 28 days.[23] Alternatively, it is possible that IGF-I excretion into the synovial fluid was reduced due to early cessation of transgene expression, death of implanted chondrocytes or maturation of the graft leading to decreased penetrability. Loss of transgene expression can occur due to cytotoxic T cell responses that destroy transduced cells. Although there did not appear to be a significant inflammatory response in treated joints, specific immune responses were beyond the scope of this experiment and should be considered as a potential cause of loss of transgene expression in future experiments. Repair tissue collected at 8 months post-implantation from the 4 treatment groups did not show any significant differences in IGF-I transcriptional activity based on qPCR which supports loss of transgene overexpression in the long-term. Biopsies of repair tissue at earlier time points could shed light on longevity of IGF-I expression, however, disruption of the repair site is a major concern.

Adeno-associated virus has been touted as an ideal viral vector due to its lack of pathogenicity, ability to transduce non-dividing cells, and prolonged expression.[17] Tissue

tropism has been shown for several AAV serotypes and these affinities are likely species-specific. rAAV5 provided excellent transduction of equine chondrocytes in this study, based on evidence from fluorescent microscopy showing GFP expression in pre-implantation monolayer cultures. Other serotypes, including rAAV2, have been evaluated for transduction efficiency of articular cells, however, preliminary data in our laboratory comparing rAAV2 and rAAV5 revealed optimal transduction of chondrocytes by rAAV5 at a dose of 10^5vg/cell.[23]

In the past, similar studies in the horse have used allogeneic chondrocytes as a source for cell-based repair.[8,12] We investigated the use of autologous chondrocytes in order to eliminate potential host immune reaction to allogeneic cells. Although autologous cells do not incite an immune response, this cell source is not without concerns. Donor site morbidity is a major problem in human ACI.[27] In our case, 1.5-2.5g of cartilage was removed from the distal talus and although these donor sites are non-weight bearing surfaces in the horse, such extensive cartilage debridement risks long-term negative effects in the joint. The expansion of chondrocytes in culture following cartilage digestion is associated with dedifferentiation and loss of the chondrocyte phenotype.[28-30] It is difficult to find an ideal balance between limiting the amount of cartilage harvested in order to decrease donor site morbidity, while simultaneously obtaining enough tissue that extensive expansion, and thereby dedifferentiation, is not required to attain adequate cell numbers for implantation. It is worth noting that redifferentiation of chondrocytes has been shown to occur rapidly in three-dimensional cultures [31] and could be expected to occur in the fibrin complexes used in this study.

The minimally invasive nature of chondrocyte implantation in this study is a major advantage of this technique. Transduced chondrocytes were able to be stably implanted into chondral defects using a self-polymerizing cell/fibrinogen mixture and calcium-activated thrombin under arthroscopic guidance. Eliminating the need for an arthrotomy to access cartilage defects greatly decreases post-operative morbidity and healing times. Although both cartilage harvest and chondrocyte implantation were performed using minimally invasive techniques, donor site morbidity and need for a second surgery are ongoing concerns. Considering the potential detrimental consequences of cartilage harvest, alternative cell sources for cartilage repair, such as chondrogenically differentiated stem cells may provide a superior treatment method. This cell source would eliminate both donor site morbidity and the need for multiple surgeries.

This well-established equine model likely provides the closest approximation to cartilage repair in man and is a more robust model than more commonly used small animal models. The cartilage of the equine lateral trochlear ridge has approximately the same thickness (2-3mm) and load bearing characteristics as human femoral condyles.[32] Inter-animal variability plays an important role in gene therapy; host differences can affect transduction efficiency, transgene expression and survival of transduced cells. In this study, we observed moderate inter-animal variability; however, the response to AAV-transduced autologous chondrocytes in man will likely be far more variable as the population is more diverse. Additionally, there appears to be significant differences in the immune response to AAV between humans and horses. For example, cytotoxic $CD8^+$ T-cell responses have been shown in humans[33] but have not been demonstrated in horses.

Although improved healing was noted in the rAAV5-IGF-I treated defects, the transition from "hyaline-like" to hyaline repair tissue remains challenging. At 8 months post-implantation graft-host integration was complete in some histological sections of treated defects, however, full integration around the entire perimeter of the defects was not observed, which would likely limit durability of the repair tissue. It is possible that longer-term evaluation of the grafts may have shown enhanced integration. Although full integration was not observed, it is noteworthy that all grafts remained stable throughout the 8 month study period during which horses had free access to exercise placing substantial load and stress on the repair tissue. rAAV5-IGF-I repair tissue had superior smoothness and collagen type II content compared to other treatments, however, the rAAV5-IGF-I neocartilage was not consistently smooth (**Figure 2.5a**) and collagen type II did not appear to be evenly distributed (**Figure 2.6a**). This may be explained by differences in transgene expression of transduced chondrocytes throughout the depth of the implant. The superficial layer of the repair tissue undergoes more intense wear post-implantation which could increase cell death and decrease ECM production.

In summary, minimally invasive implantation of autologous chondrocytes transduced *ex vivo* with rAAV5-IGF-I appears to improve long-term healing of full-thickness chondral defects in the equine model. Important features of hyaline-like repair tissue were noted in rAAV5-IGF-I treated lesions including hyaline-like tissue architecture, predominance of chondrocytes as the main cell type, and an increased abundance of collagen type II. Importantly, rAAV5 transduced chondrocytes appeared to have little adverse effect on joints as there were no significant alterations in synovial fluid composition, no significant

differences noted in synovial biopsies, and all treated horses remained clinically sound. Although this study showed improved repair with genetically-modified chondrocytes, return to native tissue continues to be a significant challenge. Further investigation into genetically-enhanced chondrocytes that concurrently limit catabolic cytokine abundance may improve the hyaline character of the repair more profoundly.

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CHAPTER THREE

CATABOLIC CYTOKINE SUPPRESSION IN CHONDROCYTES BY rAAV2 DELIVERY OF A SHORT HAIRPIN RNA INTERENCE MOTIF TARGETING INTERLEUKIN-1β

Abstract

Post-traumatic activation of the catabolic cascade plays a major role in progressive degradation of articular cartilage. Interleukin-1β (IL-1β) is a primary initiator of the catabolic axis and is significantly upregulated in chondrocytes following injury. IL-1β in turn activates key degradative enzymes, including matrix metalloproteinases (MMPs) and aggrecanases (ADAMTS), and other pro-inflammatory mediators such as PGE_2, that contribute to breakdown of the extra-cellular matrix (ECM). Loss of integrity of the ECM precipitates osteoarthritis (OA), a prevalent and irreversible disease that currently lacks any curative treatment. Post-transcriptional silencing of IL-1β, mediated by RNA interference, may offer a sustainable, effective reduction in IL-1β expression and thereby, limit the downstream degradative effects. We hypothesized that transduction of chondrocytes, using an rAAV2 vector expressing a short hairpin IL-1β RNA interference motif would lead to a significant decreases in IL-1β expression, as well as decreased expression of other catabolic enzymes including MMPs and aggrecanases. Chondrocyte monolayer cultures were transduced with rAAV2-tdT-shIL-1β in serum-free media. The fluorescent protein, tdTomato, was used to determine transduction efficiency via flow cytometry and fluorescent microscopy. Cells were stimulated with lipopolysaccharide (LPS) 48 hours following transduction. After 24 hours of LPS stimulation, supernatants were collected and cells lysed for RNA isolation and gene expression analysis. rAAV2-tdT-shIL-1β transduction of chondrocytes led to significantly decreased expression of IL-1β, TNF-α and ADAMTS-5, compared to untransduced, LPS-stimulated chondrocytes. PGE_2 synthesis was also significantly downregulated. This study showed effective silencing of IL-1β using a rAAV2 vector expressing a short hairpin IL-1β

sequence. Additionally, we found significant downstream effects of IL-1β silencing, including decreased expression of key catabolic factors, TNF-α and ADAMTS-5. Gene therapy targeting catabolic cytokines through RNA interference may provide a promising treatment modality for the OA joint.

Introduction

Osteoarthritis (OA) is a prevalent, debilitating disease that currently lacks any effective treatment and often culminates in total joint replacement surgery. It is characterized by progressive degradation of the extra-cellular matrix (ECM) leading to joint swelling, reduced mobility and pain. Chondrocytes are the sole cell type responsible for maintaining the integrity of the ECM, and disruption of chondrocyte metabolism following trauma to the articular surface is considered a pivotal step in deterioration of the osteoarthritic joint. Perturbations in gene expression patterns of chondrocytes, including increased expression of catabolic cytokines and degradative enzymes, have been shown to cause slow destruction of the ECM.[1]

Interleukin-1β (IL-1β), a pro-inflammatory cytokine, is considered a primary instigator of cartilage degradation[2-4] and is upregulated in equine OA chondrocytes and synovial fluid from OA joints.[5] IL-1β acts through autocrine and paracrine signaling pathways to increase synthesis of degradative enzymes, including MMPs and aggrecanases, that actively breakdown the ECM.[6] Simultaneously, IL-1β decreases expression of collagen type II and inhibits aggrecan synthesis.[7,8] Inflammatory mediators, such as prostaglandin E_2 (PGE_2)[9] and nitric oxide (NO),[10] are also increased by IL-1β and likely play a significant role in the pain response associated with OA.

The beneficial role of IL-1 receptor antagonist protein (IL-1Ra) has received ample attention as it decreases the degradative effects of IL-1β through competitive binding of IL-1

receptors.[11-13] A potential impediment to IL-1Ra therapy is the requirement of 100-fold, or greater, excess of IL-1Ra over IL-1 to achieve effective antagonism.[14] Maintaining sufficient IL-1Ra concentrations in the synovial fluid is difficult, especially considering the positive feedback loop exhibited by IL-1β.[15,16] Post-transcriptional silencing of IL-1β through RNA interference (RNAi) may offer a superior method of controlling the catabolic cascade involved in joint injury and OA. Small interfering RNAs (siRNAs) were originally investigated as therapeutic modalities. However, more recently viral vectors have been explored as they provide long-term, sustainable expression of short hairpin RNAs.

A gene therapy approach for treating OA is attractive as the disease often affects a single joint that can be treated locally with an intra-articular injection of a vector over-expressing a therapeutic transgene. Sustained, vector-mediated knockdown of IL-1β expression in perturbed chondrocytes may ameliorate degradation of the ECM by helping to restore normal physiologic balance in cartilage, thereby providing a therapeutic option for OA joints. Recombinant adeno-associated virus vectors (rAAV) are ideal candidates for intra-articular gene therapy as they can invade non-dividing cells, are non-pathogenic, and replication deficient.[17] Transduction of articular tissues, including chondrocytes, synoviocytes and intact cartilage, by rAAV vectors has been well-demonstrated.[18-20]

The objective of this study was to evaluate the effects of rAAV2 mediated knockdown of IL-1β on gene expression and protein synthesis in chondrocytes cultured in an OA model. We hypothesized that transduction of chondrocytes with rAAV2-shIL-1β prior to stimulation with lipopolysaccharide (LPS) would effectively silence IL-1β and would in turn decrease

expression of catabolic cytokines, degradative enzymes, and inflammatory mediators involved in the IL-1β signaling pathway. We also hypothesized that IL-1β interference would rescue suppression of key matrix proteins, including collagen type II and aggrecan, that occurs following LPS stimulation.

Materials & Methods

Tissue culture

Cartilage was harvested from the articular surface of three young Thoroughbred horses (mean age = 1.23 years) and digested in 0.075% collagenase (Worthington Biochemical, Lakewood, NJ) as previously described.[21] Following digestion, cells were filtered and centrifuged at 300 *g* for 5 minutes. Cell pellets were washed and cells counted before plating in 24-well plates (Corning Inc., Corning, NY) at a density of 1×10^5 cells/cm^2. Chondrocytes were cultured in Ham's F12 medium (Corning Inc., Corning, NY) supplemented with 10% fetal bovine serum (FBS), 50μg/ml ascorbic acid, 30μg/ml α-ketoglutarate, 300μg/ml L-glutamine, 100units/ml penicillin/streptomycin, and 25m*M* HEPES (Gibco-Life Technology, Grand Island, NY) and allowed to adhere for 48 hours. Prior to any transfection or transduction procedure, medium was replaced with serum-free Opti-MEM (Invitrogen, Grand Island, NY) for 4 hours. All chondrocyte experiments were performed in triplicate.

siRNA and plasmid screening

Three different siRNA sequences targeting IL-1β were designed by Ambion (Grand Island, NY) using their proprietary software and three siRNA sequences were designed using the online algorithm at the Public TRC Portal (www.broadinstitute.org/rnai/public/seq/search) (**Table 3.1**). These six siRNA sequences were synthesized (IDT, Coralville, IA) and evaluated for knockdown efficiency in monolayer cultures using chondrocytes from 3 different horses. Cells were transfected with siRNA in serum-free medium using DharmaFECT transfection reagent (Thermo Scientific, Waltham, MA). Following transfection, chondrocytes were stimulated with 50μg/ml E. Coli O55:B5 lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, MO) in serum-free medium. After 24 hours, cells were lysed and RNA isolated using the PerfectPure RNA kit (5 Prime, Gaithersburg, MD). The two siRNA sequences with the most profound decrease in IL-1β expression were then designed as short hairpins (sh) by addition of a loop, poly A tail, and restriction sites. The shRNA sequences were synthesized as dsDNA (IDT, Coralville, IA) and ligated into the U6 promoter-driven pSilencer 2.1-U6 Puro plasmid (Life Technologies, Grand Island, NY). Knockdown efficiency of the 2 shRNA sequences was determined in monolayer chondrocytes following transfection of cells using FuGENE HD Transfection Reagent (Promega, Madison, WI).

Table 3.1. siRNA sequences targeting IL-1β

siIL-1β Sequence Name	**Sequence**
#1	5’-CCAGUGACAUGAUGACUUA-3’
#2	5’-UCCGGGACAUAUACCAUAAAU-3’
#3	5’-GCUUCAAUUCUCCCACCAA-3’
#4	5’-GACAACUGGGAUGAUGAUUAU-3’
#5	5’-AAGUCAGUUAUGUCCCGGCCG-3’
#6	5’-CCAGUUUAAUUUGGACUAG-3’

Adeno-associated viral vector production

The most efficient shIL-1β sequence and the red fluorescent protein, tdTomato (tdT), were subcloned into the rAAV transfer plasmid pHpa-trs-SK. The transgenes were flanked by inverted terminal repeats; tdT was under control of the cytomegalovirus (CMV) early promoter and shIL-1β was under control of the U6 promoter. scAAV2-tdT-shIL-1β was generated by the Research Vector Core at The Children's Hospital of Philadelphia (CHOP) in HEK293 cells using the triple plasmid transfection method in which cells were transfected with three different plasmids. The plasmids included: 1) rAAV-tdT-shIL-1β construct, 2) the AAV *rep* and *cap* genes and 3) the adenovirus helpervirus.

AAV transduction and LPS stimulation of chondrocytes

Chondrocytes were cultured in monolayer as described above for 48 hours. Four treatment groups were evaluated: 1) no vector, no LPS (C); 2) no vector, LPS (LPS); 3) rAAV2-tdT-shIL-1β, no LPS (shIL-1β); 4) rAAV2-tdT-shIL-1β, LPS (shIL-1β+LPS). Medium was changed to serum-free Opti-MEM 4 hours prior to transduction. Chondrocytes were transduced with 1×10^5 viral genomes/cell for 2 hours at 37^oC. Transduction medium was then replaced with fresh Opti-MEM and cultures were maintained for 48 hours prior to LPS stimulation. Chondrocytes were stimulated with 50μg/ml of LPS for 24 hours prior to collection and further analysis.

Flow cytometry and fluorescent microscopy

Chondrocytes were trypsinized, washed, and suspended in 0.5% PBS/BSA 24, 48 and 72 hours post-transduction for flow cytometric analysis using a FACSCanto II flow cytometer (BD Biosciences, San Diego, CA). tdTomato expression was quantified using a 585/42 filter to measure fluorescence. Untransduced chondrocytes were used to set fluorescent gates in order to account for autofluorescence. Fluorescent microscopy, to visually assess transduction of cells, was also performed at these time points (Olympus Inverted Microscope IX73, Olympus, Center Valley, PA).

Gene expression

Chondrocytes were lysed and RNA was isolated using the PerfectPure RNA kit (5 Prime, Gaithersburg, MD). Purity and concentration of the RNA was assessed using UV microspectrophotomerty (NanoDrop 2000 Spectrophotometer, Thermo Scientific, Waltham, MA). Quantitative real-time polymerase chain reaction (qRT-PCR) was used to assess gene expression with all samples run in duplicate (ABI PRISM 7900 HT Sequence Detection System, Applied Biosystems, Foster City, CA). Equine primers and dual-labeled fluorescent probes [6-carboxyfluorescein (FAM) as the 5' label (reporter dye) and 5-carboxymethylrhodamine (TAMRA) as the 3' label (quenching dye)] were used. Sequences for primers and probes were generated from GenBank (National Institutes of Health, Bethesda, MD) or available from clones with the laboratory of Dr. Alan Nixon. Primer sequences are presented in **Table 3.2**. Total copy number of mRNA was determined using a

validated standard curve and these values were normalized to the housekeeping gene, 18S.

PGE_2 synthesis

Medium was collected from monolayer cultures 24 hours following LPS stimulation and PGE_2 concentration was quantified using a competitive enzyme immunoassay (Enzo Life Sciences, Farmingdale, NY). Optical density was measured using a Tecan microplate reader at 405nm with wavelength correction set at 570nm.

Statistics

Triplicate culture samples for n=3 horses were performed. Flow cytometry data was expressed as mean percentage ± SEM. Changes in gene expression were shown as mean percentage change ± SEM relative to LPS chondrocytes. Statistical analysis was performed using a one-way analysis of variance (ANOVA) to compare differences between the four different treatment groups. Pairwise comparisons were made using a Tukey's *post hoc* test. Statistical analysis was performed using JMP. The level of significance was set at $p<0.05$.

Table 3.2. Equine primer sequences used to analyze gene expression

Gene	Forward Primer Sequence	Reverse Primer Sequence	Probe Sequence
18S	5'-CGGCTTTGGTGACTCTAGATAACC-3'	5'-CCATGGTAGGCACAGCGACTA-3'	5'- TGGCGTTCAGCCACCCGAGATT-3'
IL-1β	5'-CGTCTCCCAGAGCCAATCC-3'	5'-CACCAGGCTGACTTTGAGTGAGT-3'	5'-CCCTCTGCTGAGCAGGGCCTCTC-3'
TNF- α	5'-CAGCCGCTTAGCTGTCTCCTA-3'	5'-GTGTGGCAAGGGCTCTTGAT-3'	5'-CCGTCCAAGGTCAACCTCCTCTCTGC-3'
ADAMTS-4	5'-CCCTGGTCTCCGAAACCTCTA-3'	5'-TATTCACCATTGAGGGCATAGGA-3'	5'-CTGGCCCTGAAGCTCCCAGATGG-3'
ADAMTS-5	5'- CAGACGTTGGGACCATATGCT-3'	5'-TGCGTGGAGGCCATCAT-3'	5'-AGAGCGCAGCTGTGCTGTGATTGAA-3'
MMP-3	5'-CTTATCAAAAATGGCTGCGTCTATT-3'	5'-GCAGAGACAGTGTTTTCATTTTTAAG-3'	5'- CCTCTCGGGTAACCCGCTTGTTCC-3'
MMP-13	5'-TGAAGACCCGAACCCTAAACAT-3'	5'-GAAGACTGGTGATGGCATCAAG-3'	5'-CAAAACACCAGACAAATGCGATCCTTCCTTA-3'
IL-6	5'-AGTAACCACCCCTGACCCAACT-3'	5'-TGTTGTGTTCTTCAGCCACTCA-3'	5'-CCTGCTGGCTAAGCTGCATTCACAGA-3'
RelA (p65)	5'-GCTTATGGATTCTGAGGGTGTGT-3'	5'-CCAAAAGGATATAGATACTGCCAATAAA-3'	5'-CCAACTGCCCCCAACTTCTTTGGG-3'
IL-10	5'-CTTGTCGGAGATGATCCAGTTTT-3'	5'-AGTTCACGTGCTCCTTGATGTCT-3'	5'-ATGCCCCAGGCTGAGAACCACG-3'
Collagen type II	5'-CGCTGTCCTTCGGTGTCA-3'	5'-CTTGATGTCTCCAGGTTCTCCTT-3'	5'-TCCGGCAGCCAGGACCGAA-3'
Aggrecan	5'-GATGCCACTGCCACAAAACA-3'	5'-GATGCCACTGCCACAAAACA-3'	5'-CCGAGGGTGAAGCTCGAGGCAA-3'

Results

siRNA screening and vector development

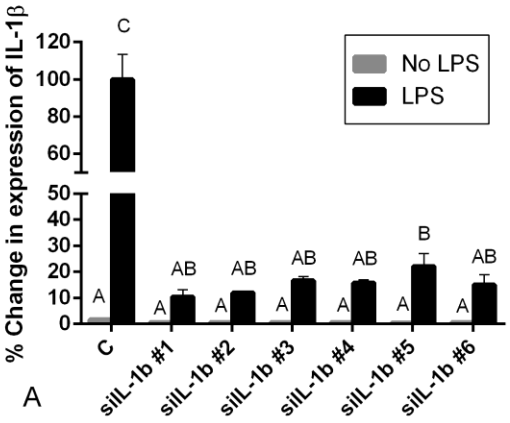
Six different sequences targeting IL-1β were evaluated for knockdown efficiency in chondrocyte monolayer cultures. All 6 sequences led to significantly decreased expression of IL-1β 24 hours following stimulation with LPS (50μg/ml) (**Figure 3.1a**), however, sequence #1 and #2 showed slightly superior silencing. Both sequences showed efficient IL-1β silencing following ligation into the plasmid pSilencer and transfection of cells using Fugene HD (**Figure 3.1b**). Sequence #1 appeared to have superior silencing compared to #2 (**Figure 3.1b**); therefore, sequence #1 was selected as the shRNA to be expressed by the rAAV vector. Continued efficient knockdown of IL-1β was observed following ligation of shIL-1β #1 into the AAV transfer plasmid that was designed to co-express tdTomato (**Figure 3.1c)**.

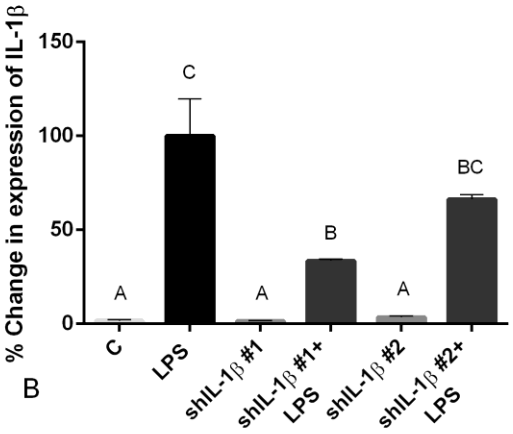
Transduction efficiency of rAAV2-tdT-shIL-1β in equine chondrocytes cultured in monolayer

The red fluorescent protein, tdTomato, was packaged into the rAAV2 vector in order to assess transduction efficiency. Equine chondrocytes cultured in monolayer were efficiently transduced with the rAAV2-tdT-shIL-1β vector, as determined by flow cytometry quantifying expression of tdTomato (**Figure 3.2a-c**). The percentage of cells (± SEM) expressing tdTomato increased throughout the 72 hour culture period following transduction. At 24 hours post-transduction, 16±1.66% of cells were positive; at 48 hours post-transduction, 31.9±2.0% of cells were positive; and at 72 hours post-transduction 42.2±1.44% of cells were

positive for the reporter protein (**Figure 3.2b,c**). Fluorescence microscopy images taken at similar time points supported the flow cytometry findings (**Figure 3.3**). The number of cells expressing tdTomato increased over the 72 hour culture period.

Figure 3.1. Changes in expression of IL-1β in chondrocytes 24 hours following stimulation with LPS (50μg/ml). % Change (±SEM) is shown relative to control chondrocytes treated with LPS. (a) Comparison of six different siRNA targeting sequences, all of which show significant decrease in IL-1β expression. (b) IL-1β expression in LPS-stimulated chondrocytes following transfection with pSilencer expressing shIL-1β #1 and shIL-1β #2. (c) IL-1β expression in LPS-stimulated chondrocytes following transfection with the rAAV transfer plasmid co-expressing tdTomato. Different letters denote significant differences between groups as determined using ANOVA followed by Tukey's post-hoc analysis. $p<0.05$


% Change in expression of IL-1β
No LPS
LPS
C
siIL-1b #1
siIL-1b #2
siIL-1b #3
siIL-1b #4
siIL-1b #5
siIL-1b #6
A


% Change in expression of IL-1β
C
LPS
shIL-1β #1
shIL-1β #1+ LPS
shIL-1β #2
shIL-1β #2+ LPS
B

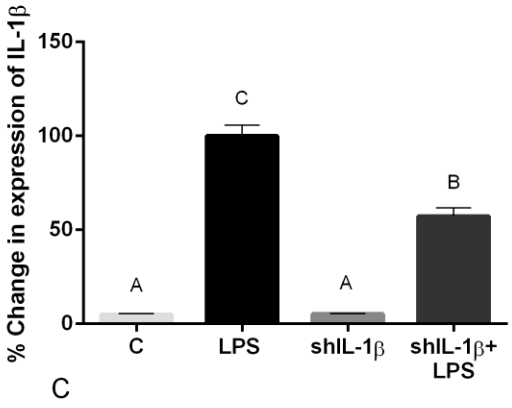
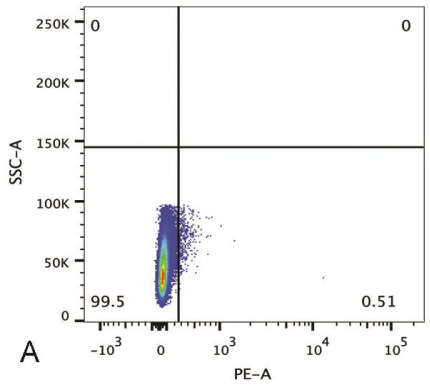
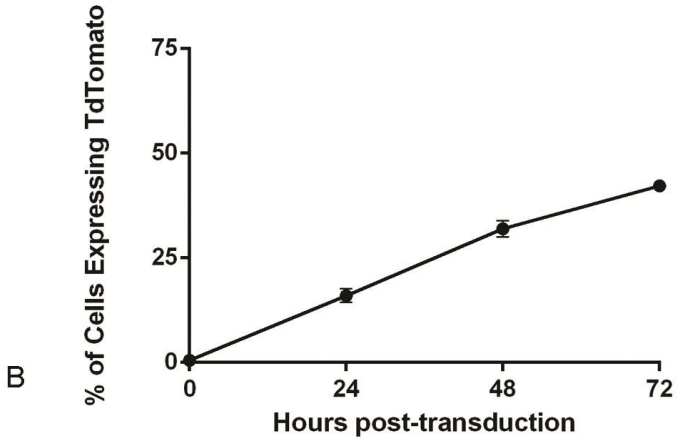

% Change in expression of IL-1β
C
LPS
shIL-1β
shIL-1β+ LPS
C

Figure 3.2. Chondrocytes cultured in monolayer and transduced with the rAAV2-tdT-shIL-1β vector. Fluorescence was quantified using flow cytometry and visually assessed using fluorescence microscopy. (a) Representative flow cytometric plot of untransduced chondrocytes used to set fluorescent gates to account for autofluorescence of chondrocytes. (b) Transduction efficiency assessed by % of chondrocytes (±SEM) expressing the fluorescent protein, tdTomato 24, 48 and 72 hours post-transduction. (c) Representative flow cytometric plots of transduced chondrocytes 24, 48 and 72 hours following transduction.


A
SSC-A
PE-A
0
0
99.5
0.51


B
% of Cells Expressing TdTomato
Hours post-transduction
0
25
50
75
0
24
48
72

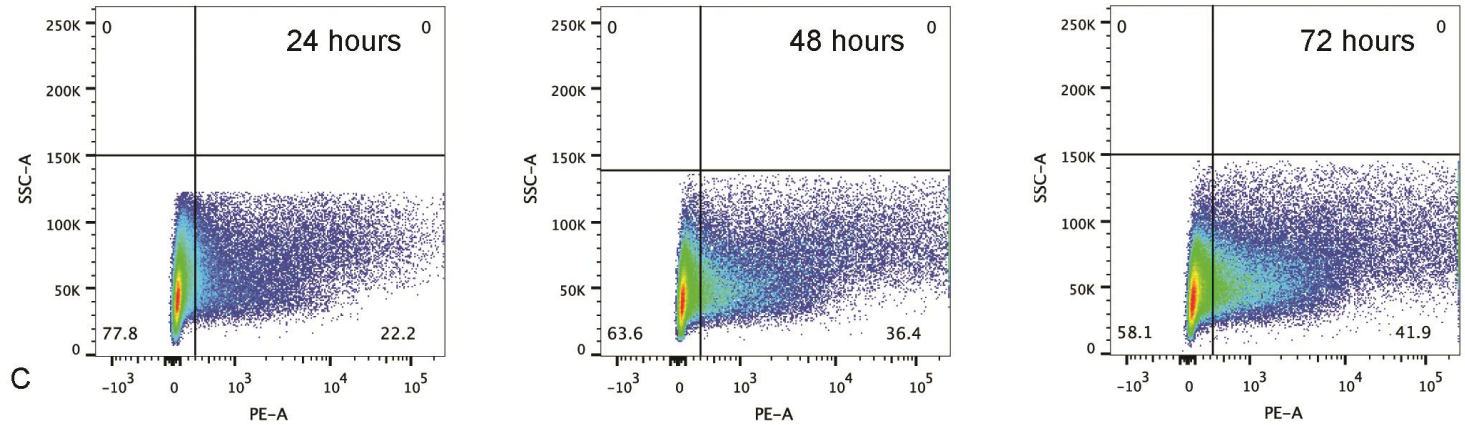
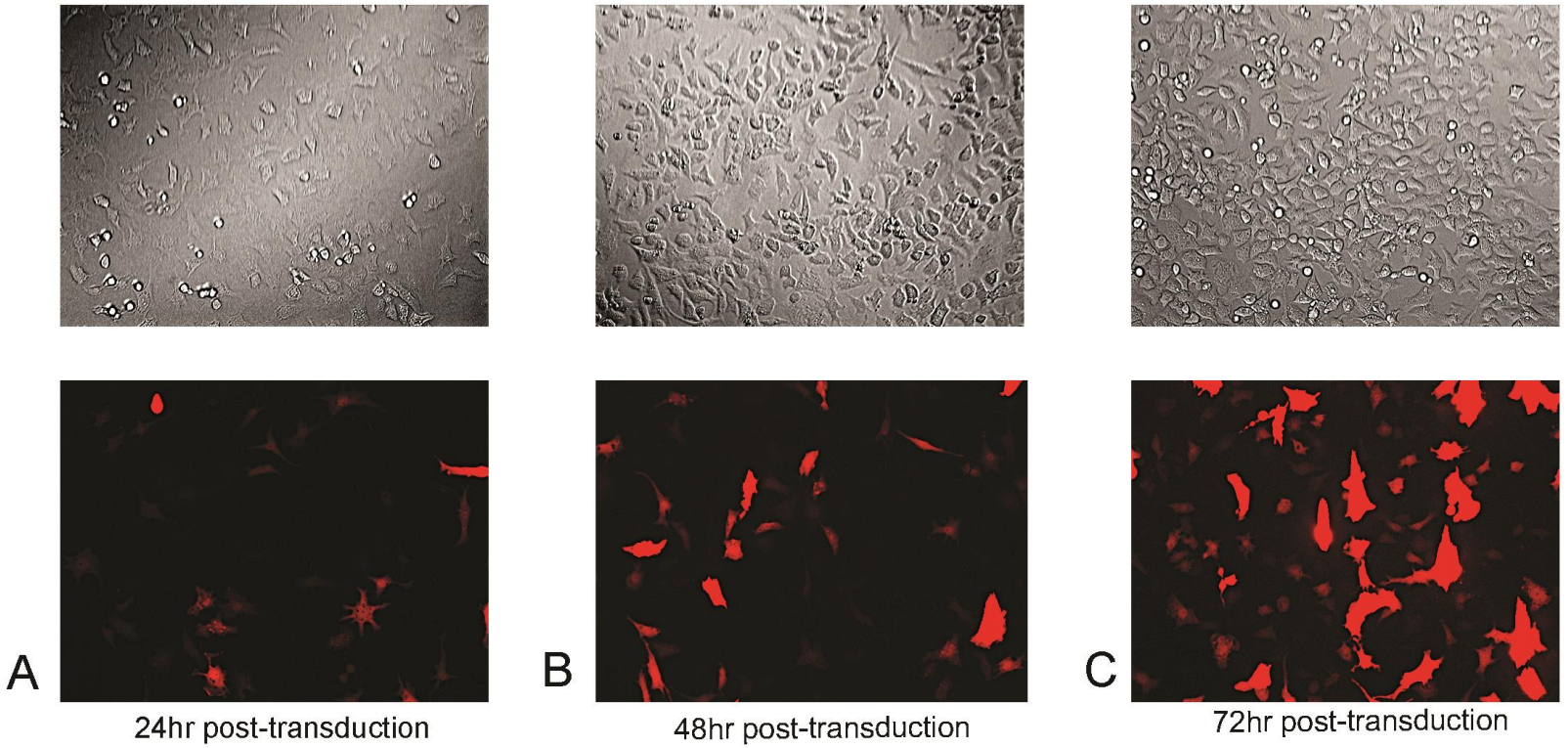

C
24 hours
48 hours
72 hours
SSC-A
PE-A
77.8
22.2
63.6
36.4
58.1
41.9

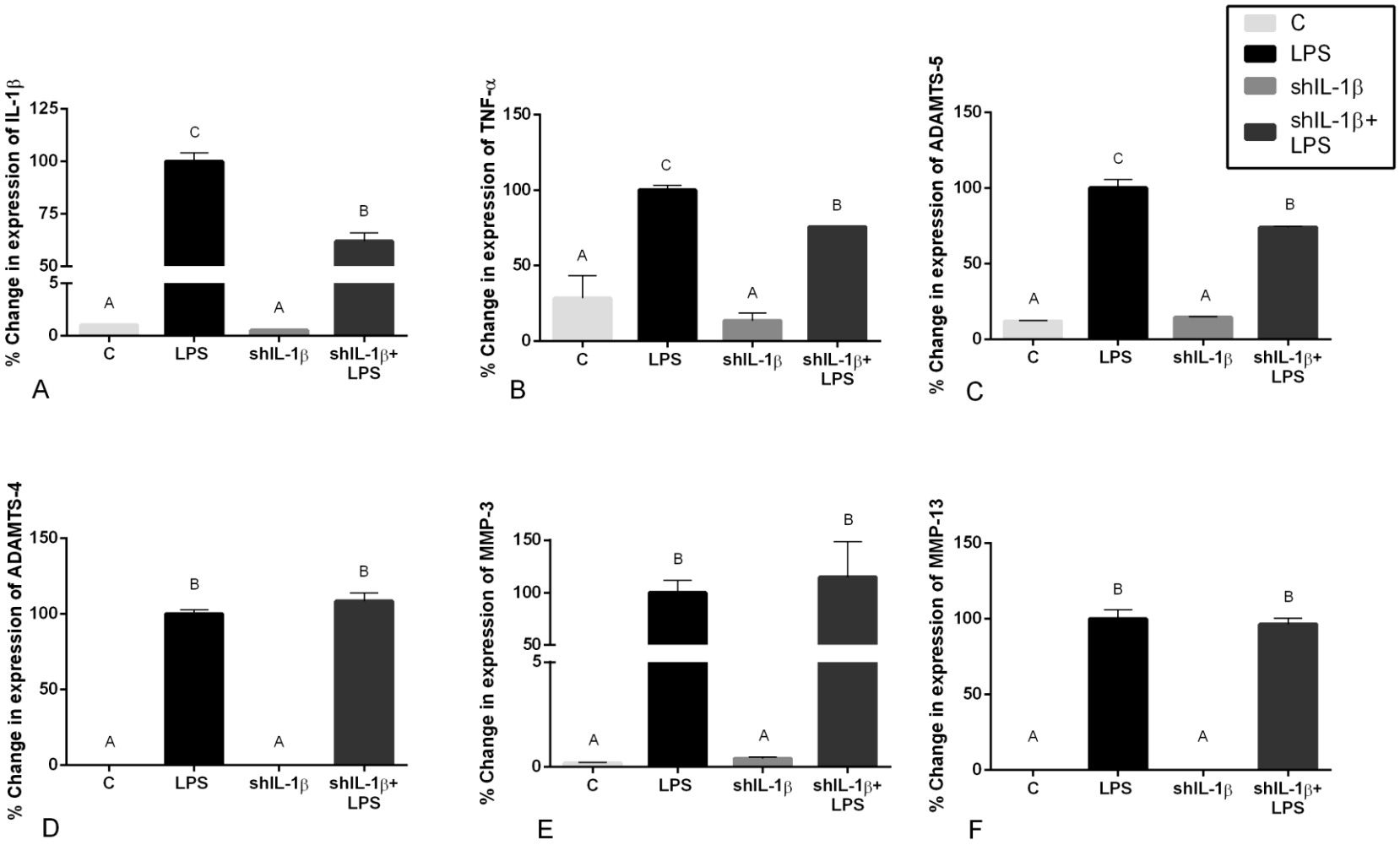
Figure 3.3. Representative bright field (top) and fluorescence microscopy (bottom) images of chondrocytes cultured in monolayer (a) 24 hours, (b) 48 hours, and (c) 72 hours post-transduction with rAAV2-tdT- shIL-1β. Fluorescence intensity and number of cells expressing the fluorescent protein increased during the 72 hour culture period. Magnification, 20x.


A
B
C
24hr post-transduction
48hr post-transduction
72hr post-transduction

Effect of rAAV2-tdT-shIL-1β transduction on expression of catabolic cytokines and degradative enzymes

Expression of catabolic cytokines and degradative enzymes, including IL-1β, tumor necrosis factor-α (TNF-α), MMP-3, MMP-13, ADAMTS-4, and ADAMTS-5, was evaluated in rAAV2-tdT-shIL-1β transduced and untransduced chondrocyte cultures 24 hours following LPS stimulation. Changes in expression of all genes were evaluated relative to LPS stimulated chondrocytes (LPS). All catabolic cytokines and enzymes were significantly increased in LPS cultures compared to cultures that received no LPS stimulation (**Figure 3.4**). IL-1β expression was significantly decreased in shIL-1β+LPS chondrocytes compared to LPS chondrocytes, 24 hours following LPS stimulation ($p<0.0001$) (**Figure 3.4a**) consistent with IL-1β RNA interference. IL-1β expression was reduced by approximately 42% in shIL-1β+LPS chondrocytes but remained significantly higher than C and shIL-1β chondrocytes. TNF-α expression was also significantly reduced in shIL-1β+LPS chondrocytes compared to LPS chondrocytes, 24 hours following LPS stimulation ($p=0.017$) (**Figure 3.4b)** as was expression of ADAMTS-5 ($p=0.0001$) (**Figure 3.4c**). There were no significant differences in expression of MMP-3, MMP-13, or ADAMTS-4 in shIL-1β+LPS chondrocytes and LPS chondrocytes (**Figure 3.4d-f**).

Figure 3.4. Quantitative polymerase chain reaction (qPCR) data showing % change in gene expression of catabolic cytokines and degradative enzymes in control (C), untransduced, LPS stimulated (LPS), rAAV2-tdT-shIL-1β transduced (shIL-1β) and rAAV2-tdT-shIL-1β transduced, LPS stimulated (shIL-1β+LPS) chondrocytes. % Change (±SEM) is shown relative to LPS chondrocytes. shIL-1β+LPS chondrocytes had significantly decreased (a) IL-1β, (b) TNF-α and (c) ADAMTS-5 expression compared to LPS chondrocytes. Expression of (d) ADAMTS-4, (e) MMP-3 and (f) MMP-13 showed no significant differences between shIL-1β+LPS and LPS chondrocytes. Data were analyzed using ANOVA followed by Tukey's post-hoc analysis. $p<0.05$

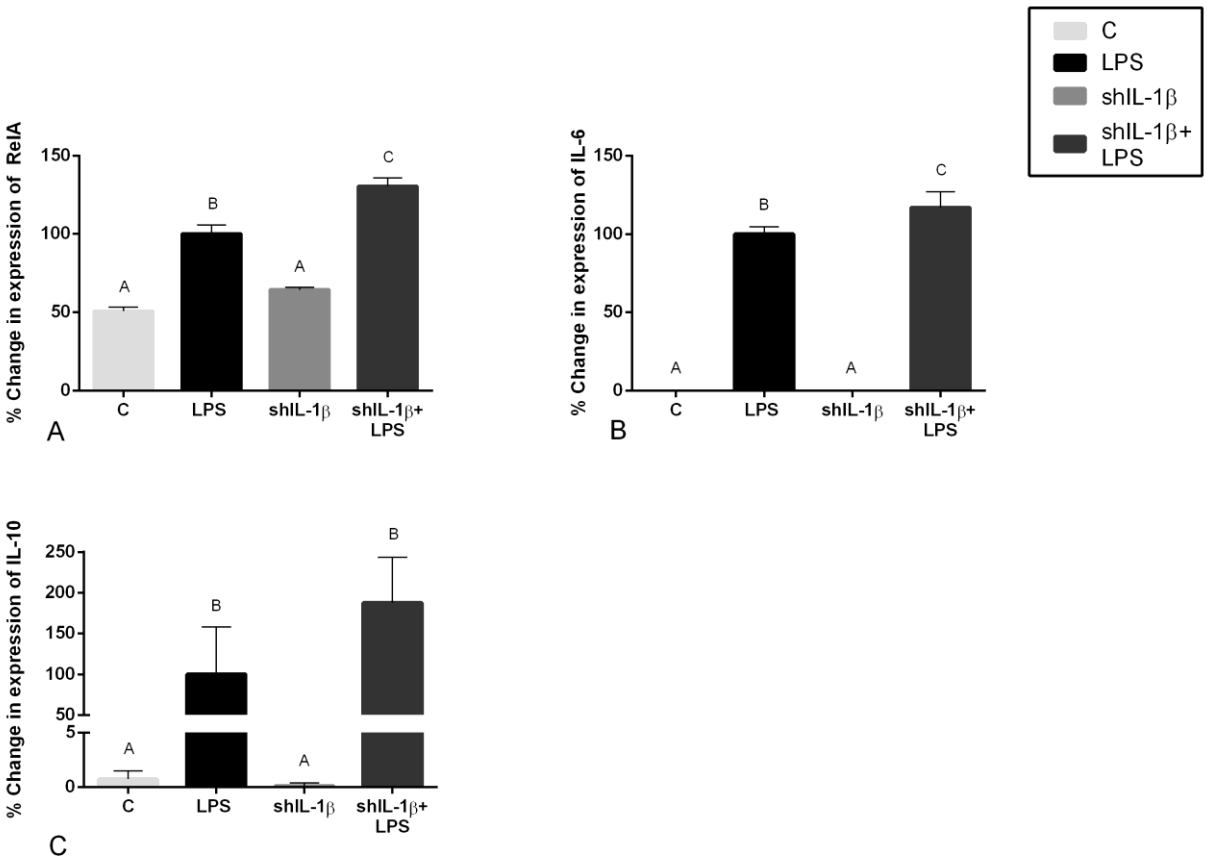

% Change in expression of IL-1β
125
100
75
50
5
0
A
C
A
B
C
LPS
shIL-1β
shIL-1β+ LPS
A
% Change in expression of TNF-α
150
100
50
0
A
C
A
B
C
LPS
shIL-1β
shIL-1β+ LPS
B
% Change in expression of ADAMTS-5
150
100
50
0
A
C
A
B
C
LPS
shIL-1β
shIL-1β+ LPS
C
C
LPS
shIL-1β
shIL-1β+ LPS
% Change in expression of ADAMTS-4
150
100
50
0
A
B
A
B
C
LPS
shIL-1β
shIL-1β+ LPS
D
% Change in expression of MMP-3
150
100
50
5
0
A
B
A
B
C
LPS
shIL-1β
shIL-1β+ LPS
E
% Change in expression of MMP-13
150
100
50
0
A
B
A
B
C
LPS
shIL-1β
shIL-1β+ LPS
F

Effect of rAAV2-tdT-shIL-1β transduction on expression of pro-inflammatory mediators

In addition to the major catabolic cytokines and degradative enzymes in articular cartilage, changes in expression of several inflammatory mediators were evaluated. Expression of RelA (p65), a major subunit in the NF-κB family of transcription factors, was significantly increased by LPS stimulation in both LPS and shIL-1β+LPS chondrocytes compared to unstimulated chondrocytes (**Figure 3.5a**). Additionally, RelA (p65) expression in shIL-1β+LPS chondrocytes was even higher than in LPS chondrocytes ($p=0.005$). IL-6 expression was significantly increased by LPS stimulation in both untransduced and transduced chondrocytes (**Figure 3.5b**). Similar to the pattern seen with RelA (p65), IL-6 expression in shIL-1β+LPS chondrocytes was significantly higher than that of LPS chondrocytes ($p=0.01$). Finally, expression of IL-10 was significantly increased by LPS stimulation (**Figure 3.5c**). shIL-1β+LPS chondrocytes had the highest expression of IL-10, however, this was not significantly different than LPS chondrocytes.

Supernatants from chondrocyte cultures were collected 24 hours following LPS stimulation and PGE_2 concentrations were quantified using ELISA. PGE_2 was significantly increased in LPS stimulated chondrocytes compared to unstimulated chondrocytes. However, PGE_2 was significantly decreased in shIL-1β+LPS chondrocytes compared to LPS chondrocytes ($p=0.029$) (**Figure 3.6**).

Figure 3.5. Quantitative polymerase chain reaction (qPCR) data showing % change in gene expression of inflammatory mediators in control (C), untransduced, LPS stimulated (LPS), rAAV2-tdT-shIL-1β transduced (shIL-1β), and rAAV2-tdT-shIL-1β transduced, LPS stimulated (shIL-1β+LPS) chondrocytes. % Change (±SEM) is shown relative to LPS chondrocytes. Expression of (a) RelA (p65), (b) IL-6 and (c) IL-10 was significantly increased with LPS stimulation. The expression of both (a) RelA and (b) IL-6 were highest in shIL-1β+LPS chondrocytes. Data were analyzed using ANOVA followed by Tukey's post-hoc analysis. $p<0.05$


C
LPS
shIL-1β
shIL-1β+ LPS
% Change in expression of RelA
150
100
50
0
A
B
A
C
C
LPS
shIL-1β
shIL-1β+ LPS
A
% Change in expression of IL-6
150
100
50
0
A
B
A
C
C
LPS
shIL-1β
shIL-1β+ LPS
B
% Change in expression of IL-10
250
200
150
100
50
5
0
A
B
A
B
C
LPS
shIL-1β
shIL-1β+ LPS
C

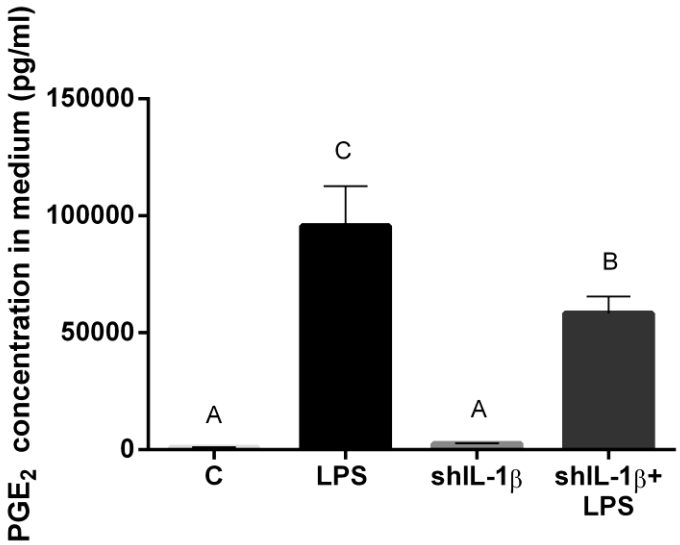
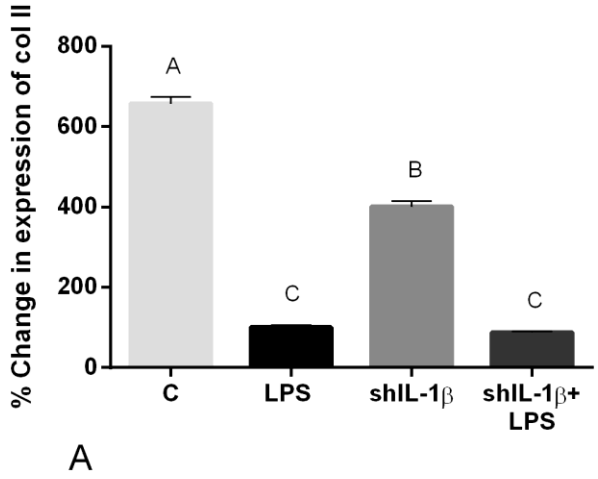


Figure 3.6. PGE_2 concentration in medium collected from chondrocyte cultures 24 hours after stimulation with LPS. Chondrocytes transduced with rAAV2-tdT-shIL-1β had significantly decreased PGE_2 in the medium compared to LPS chondrocytes. $p<0.05$

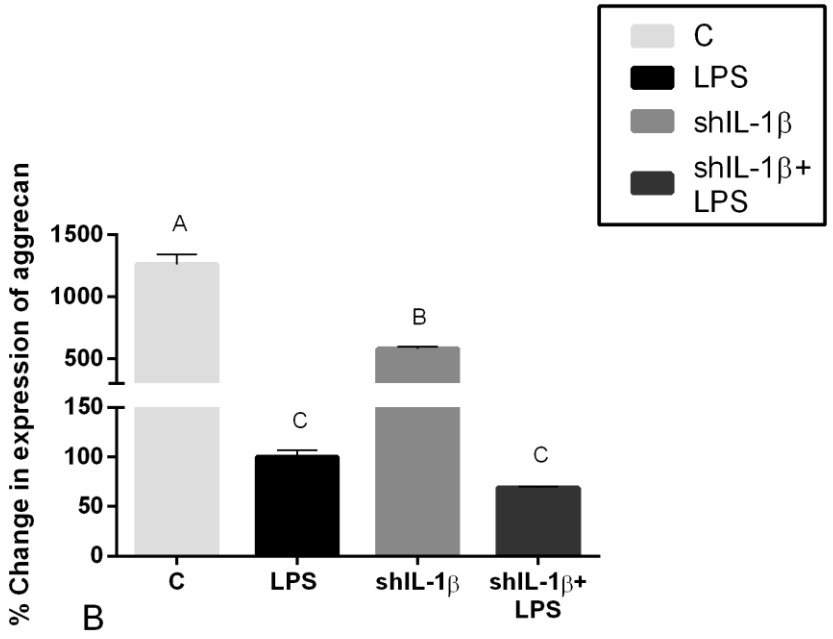
Effect of rAAV2-tdT-shIL-1β transduction on expression of anabolic factors

Expression of anabolic factors, including aggrecan and collagen type II, was also evaluated in rAAV2-tdT-shIL-1β transduced and untransduced chondrocyte cultures 24 hours following LPS stimulation. Changes in expression of all genes were evaluated relative to LPS chondrocytes. LPS stimulation caused significant suppression of both collagen type II and aggrecan expression (**Figure 3.7a,b**). Expression of collagen type II was highest in C chondrocytes (**Figure 3.7a**). Interestingly, collagen type II expression was suppressed in shIL-1β chondrocytes that were transduced only (no LPS stimulation) compared to C chondrocytes ($p<0.0001$) (**Figure 3.7a**). Additionally, the suppression of collagen type II in LPS chondrocytes was not rescued by IL-1β silencing. A similar pattern was noted in expression of aggrecan; shIL-1β chondrocytes had lower aggrecan expression than C chondrocytes ($p<0.0001$) (**Figure 7b**). Again, aggrecan suppression seen in LPS chondrocytes was not rescued by IL-1β silencing.

Figure 3.7. Quantitative polymerase chain reaction (qPCR) data showing % change in gene expression of ECM proteins in control (C), untransduced, LPS stimulated (LPS), rAAV2-tdT-shIL-1β transduced (shIL-1β), and rAAV2-tdT-shIL-1β transduced, LPS stimulated (shIL-1β+LPS) chondrocytes. % Change (±SEM) is shown relative to LPS chondrocytes. (a) Collagen type II and (b) aggrecan are shown. Both collagen type II and aggrecan expression are decreased with LPS stimulation and this is not rescued with rAAV2-tdT-shIL-1β transduction. Data were analyzed using ANOVA followed by Tukey's post-hoc analysis. $p<0.05$


% Change in expression of col II
800
600
400
200
0
A
C
B
C
C
LPS
shIL-1β
shIL-1β+ LPS

A


C
LPS
shIL-1β
shIL-1β+ LPS
% Change in expression of aggrecan
1500
1000
500
150
100
50
0
A
C
B
C
C
LPS
shIL-1β
shIL-1β+ LPS

B

Discussion

Transduction of equine chondrocytes in monolayer with the rAAV2-tdT-shIL-1β vector led to effective post-transcriptional silencing of IL-1β following stimulation with LPS. To the authors' knowledge, this is the first report of knockdown of a pivotal catabolic cytokine in the pathogenesis of OA, in equine chondrocytes, using a rAAV vector expressing a shRNA targeting IL-1β. IL-1β interference also had significant downstream effects on a major degradative enzyme, ADAMTS-5, and catabolic cytokine, TNF-α. Additionally, supernatants from LPS-stimulated chondrocytes transduced with rAAV2-tdT-shIL-1β had significantly less PGE_2 than untransduced, LPS-stimulated chondrocytes. Despite alterations in several factors involved in degradation of the ECM and pathogenesis of OA, IL-1β interference did not rescue suppression of ECM components, including collagen type II and aggrecan, observed following LPS stimulation.

The self-complementary rAAV2-tdT-shIL-1β vector was able to effectively transduce chondrocyte cultures. As expected, transduction efficiency increased over the 72 hour culture period, although at 72 hours following transduction, only 42% of cells were tdTomato+. Similar studies evaluating rAAV2 mediated transduction of chondrocytes have shown higher efficiencies (~96%) 7 days following transduction;[22] therefore, it is likely that the percentage of cells expressing tdTomato would have continued to increase past the 72 hour culture period used in this study. Cells were stimulated with LPS 48 hours post-transduction when approximately 30% of cells were positive for expression of tdTomato which may explain the only moderate knockdown (~40%) of IL-1β observed. Chondrocytes have been shown to

quickly dedifferentiate when cultured in monolayer;[23] therefore, it can be difficult to establish an ideal culture length in which chondrocytes maintain their phenotype but are also able to reach peak transduction.

By targeting IL-1β, a key cytokine in the catabolic cascade, the degradative enzyme (ADAMTS-5) and catabolic cytokine (TNF-α) activated by IL-1β were simultaneously decreased. Although both MMPs and aggrecanases are involved in breakdown of the ECM, aggrecanases likely play a more dominant role.[24] Within the aggrecanase family, ADAMTS-5 has been shown to be the major aggrecanase in murine and equine cartilage degeneration.[25,26] Decreased expression of TNF-α in rAAV2-tdT-shIL-1β transduced chondrocytes is also encouraging because, along with IL-1β, TNF-α plays a central role in joint inflammation and degradation.[27] We also found that IL-1β knockdown led to significantly less production of PGE_2, an inflammatory mediator that contributes to ECM breakdown.[28] Unfortunately, similar decreases in other downstream factors including MMP-3, MMP-13 and ADAMTS-4 were not found with IL-1β knockdown. The differences in downstream effects following IL-1β knockdown likely reflects the complex regulatory pathways at play. Although the catabolic cytokines and degradative enzymes evaluated in this study are closely related in the OA joint, each gene is individually regulated within the cell and different responses to shRNA treatment can be expected.

Although rAAV2-tdT-shIL-1β led to encouraging changes in several important mediators of OA, the vector was also associated with a pro-inflammatory response. NF-κB is a dimeric transcription complex made of several different proteins and is a central regulator of

cellular inflammation.[29] RelA (p65) is a major subunit involved in NF-κB activation which causes upregulation in expression of several proinflammatory cytokines including IFN-γ, TNF-α, and IL-6.[30] In this study, LPS stimulation led to significant upregulation of RelA (p65) expression. Intriguingly, RelA (p65) expression was even higher in shIL-1β+LPS chondrocytes compared to LPS chondrocytes. A similar pattern was seen for IL-6. Such alterations in these pro-inflammatory genes were not observed in transduced chondrocytes that were not stimulated with LPS which suggests a synergistic effect of rAAV2-tdT-shIL-1β transduction and LPS stimulation. Possible explanations for increased expression of RelA (p65) and IL-6 include an immune response to AAV or to the shRNA that is produced. Both AAV and shRNA duplexes are capable of activating an intracellular immune response through TLR9 or TLR7 signaling, respectively.[31,32] Additionally, off-target silencing and undesirable alterations in gene expression have been shown to be a side effect of shRNA treatment.[33] Evaluation of an AAV vector with a non-targeting shRNA sequence and an AAV vector without a shRNA sequence would have provided important information regarding the cellular immune response. Temporal gene expression patterns as well as a more complete evaluation of the transcriptome would also shed light on the effects of rAAV2-tdT-shIL-1β transduction in equine chondrocytes. It is important to note that although increases in RelA (p65) expression suggest activation of the NF-κB pathway, further evidence including phosphorylation of RelA (p54) and nuclear translocation of the complex are needed to confirm this. Detection of phosphorylated RelA (p65) in nuclear extracts can be accomplished using Western blot analysis.

Knockdown of IL-1β in stimulated chondrocytes failed to rescue the suppression of matrix proteins caused by LPS. Expression of aggrecan and collagen type II, the major components of the ECM, was significantly decreased following LPS stimulation and this effect was not altered by rAAV2-tdT-shIL-1β transduction. In fact, transduction alone appeared to decrease production of ECM proteins. It is possible that an intracellular immune response to the rAAV vector and/or the shRNA sequence led to these changes; however, further experiments are required to elucidate these potential responses.

A major limitation of this study is the lack of an assay to detect IL-1β protein levels following IL-1β silencing. Despite evaluation of several commercially available monoclonal antibodies against IL-1β, we were unable to find an antibody that would cross-react with equine IL-1β at the levels synthesized in these experiments. Equine IL-1β is a seemingly minimally antigenic protein in mice, which limits the ability to develop an equine specific monoclonal antibody. Although changes in gene expression are evident, the results of this study would be strengthened by further evidence of ligand synthesis via ELISA or Western blot analysis.

In summary, rAAV2-mediated post-transcriptional silencing of IL-1β helps protect chondrocytes following stimulation with LPS, a common *in vitro* model of OA. Transduction of chondrocytes in monolayer with the rAAV2-tdT-shIL-1β vector led to decreased expression of IL-1β which in turn downregulated expression of ADAMTS-5 and TNF-α, and decreased production of PGE_2. The apparent increase in the inflammatory mediators RelA (p65) and IL-6 in rAAV2-tdT-shIL-1β transduced chondrocytes stimulated with LPS

compared to untransduced chondrocytes stimulated with LPS is a concern and warrants further investigation. Overall, the results in this study suggest that RNAi may be a feasible approach for manipulating the transcriptome in chondrocytes following injury.

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CHAPTER 4

HUMORAL AND CELL-MEDIATED IMMUNE RESPONSE, AND GROWTH FACTOR SYNTHESIS, AFTER DIRECT INTRA-ARTICULAR INJECTION OF rAAV2-IGF-I AND rAAV5-IGF-I IN THE EQUINE MIDDLE CARPAL JOINT

Abstract

Intra-articular (IA) administration of viral vectors expressing a therapeutic transgene is an attractive treatment modality for osteoarthritis (OA) as the joint can be treated as a contained unit. Recombinant adeno-associated viruses (rAAV) are useful vectors as they lack pathogenicity and have long-term transgene expression. Although once considered minimally immunogenic, concerns regarding the humoral and cell-mediated immune response to AAV have been recently expressed. *In vivo* transduction of articular tissues has been investigated, however, the immune response to IA vectors remains largely unknown. We hypothesized that IA rAAV2 and rAAV5 overexpressing insulin-like growth factor-I (IGF-I) would result in long-term IGF-I formation but would also induce neutralizing antibody (NAb) and cytotoxic CD8+ T-cell responses. Twelve healthy horses were assigned to treatment (rAAV2 or rAAV5) or control (saline) groups. Middle carpal joints were injected with 5×10^{11}vg/joint. Synovial fluid was analyzed for changes in composition, NAb titers, immunoglobulin isotypes, pro-inflammatory cytokines and IGF-I. Serum was analyzed for antibody titers and cytokines. A T-cell re-stimulation assay was used to assess T-cell responses. Injection of rAAV2- or rAAV5-IGF-I did not induce greater inflammation compared to saline. Synovial fluid IGF-I from rAAV5-IGF-I treated joints was significantly increased by day 14 and remained elevated until day 56. IGF-I in rAAV2-IGF-I treated joints was only significantly increased on day 56. A capsid specific T-cell response was not noted although all virus treated horses had increased NAb in serum and synovial fluid following treatment. Overall, IA injection of rAAV2- or rAAV5-IGF-I did induce a humoral immune response but did not incite a clinically detectable inflammatory or cell-mediated immune response. The

overexpression of anabolic cytokines such as IGF-I using minimally immunogenic viral vectors represents a clinically relevant tool for treatment of articular disorders including OA.

Introduction

Traumatic joint injury is common, and due to the poor intrinsic healing capabilities of cartilage, often precipitates osteoarthritis (OA).[1] Currently there are no disease-modifying treatments for cartilage damage or OA and end-stage arthritic joints often require total joint replacement surgery. Gene therapy approaches to enhance cartilage repair and treat OA have great potential as vectors carrying transgenes can be injected intra-articularly for concentrated, local therapeutic protein production. Previous studies have shown that articular tissues, including synoviocytes and chondrocytes, can be transduced through direct IA injection.[2-4] Gene therapy techniques, which provide long-term *in situ* expression of repair-enhancing genes, would be superior to repeated injections or depots of peptide that are transient.

Insulin-like growth factor-I (IGF-I) has been shown to have anabolic and mitogenic effects on chondrocytes with increased production of extra-cellular matrix (ECM) proteins including collagen type II and aggrecan.[5] IGF-I has also been shown to enhance the repair potential of chondrocytes [6] and aids in the protection and recovery of the ECM following damage [7]. Intra-articular overexpression of IGF-I following cartilage damage could aid in chondrocyte-mediated repair of the articular surface thereby limiting joint degeneration and OA.

Many different viral vectors including retrovirus,[8] lentivirus,[9] and adenovirus [2] have been investigated for use in OA. However, a significant impediment has been the substantial

immune response which limits transduction and transgene expression [2]. Adeno-associated virus (AAV) may be a promising alternative as it is non-pathogenic, invades dividing and non-dividing cells and has long-term transgene expression.[10] Many AAV serotypes exist with wide tropism that appears to be both tissue and species specific; both AAV2 and AAV5 have been shown to effectively transduce equine synoviocytes and chondrocytes in our laboratory [11] and by others.[12]

Although AAV has great potential as a viral vector for gene therapy, the immune response to capsid proteins has surfaced as a major obstacle to its success. The humoral response has been well-documented and it is estimated that 20-40% of humans have neutralizing antibody titers against any given serotype.[13,14] Prevalence of NAbs depends on serotype with 80% of humans having neutralizing antibodies to AAV2.[15] Several animal studies have shown that titers as low as 1:2 - 1:4 can prevent successful transduction.[16-18] The presence of NAbs in synovial fluid has been shown and may limit IA gene transfer.[19,20] Although the immune response to AAV appears primarily humoral, a cellular immune response to epitopes present on the AAV capsid has been recently elucidated in several studies.[21-24] There is minimal viral gene expression in rAAV, but capsid proteins can be presented by MHC I in transduced cells following viral uncoating and processing.[25] Cytotoxic $CD8^+$ T cells that recognize capsid proteins loaded into MHC I would be able to eliminate transduced cells.

In this study, we aimed to investigate both the humoral and T-cell response to IA administration of rAAV2 and rAAV5, quantify the production of the therapeutic transgene

IGF-I, and correlate the immune response with transgene expression. We hypothesized that rAAV2- and rAAV5-IGF-I would lead to minimal joint inflammation following injection and would lead to increased levels of IGF-I in the synovial fluid. However, IGF-I concentrations would be limited by the presence of pre-existing neutralizing antibodies. Additionally, we hypothesized that a T-cell response would be invoked by re-stimulation of T cells with the AAV serotype to which the horse has been previously exposed.

Materials & Methods

Adeno-associated virus vector preparation

Full-length equine IGF-I cDNA was amplified by PCR. Equine IGF-I cDNA was subcloned into the rAAV transfer plasmid pHpa-trs-SK with SacII and NotI sites. The transgenes were flanked by inverted terminal repeats and under control of the CMV promoter. Self-complementary rAAV2-IGF-I and rAAV5-IGF-I vectors were generated by the Research Vector Core at The Children's Hospital of Philadelphia (CHOP) in HEK293 cells using the triple plasmid transfection method in which cells were transfected with three different plasmids. The plasmids included: 1) rAAV-IGF-I construct, 2) the AAV *rep* and *cap* genes and 3) the adenovirus helpervirus.

Horses

All animal procedures were approved by the Institutional Laboratory Animal Care and Use Committee at Cornell University. Twelve, healthy adult horses (6 geldings, 6 mares) between the ages of 5-19 years (median age=12.5 years) were assigned to one of 3 treatment groups: 1) Dulbecco's phosphate-buffered saline (DPBS) injection, 2) rAAV2-IGF-I, or 3) rAAV5-IGF-I, with 4 horses per group. Both middle carpal (MC) joints of each horse were used and were randomly assigned to either treatment (viral vector) or saline control. The DPBS injected group had both joints injected with DPBS in order to serve as a viral-free control group for evaluation of the systemic response to IA AAV injection. There were 7 Thoroughbreds, 1 Quarter Horse and 3 Warmbloods.

Intra-articular injection

Intra-articular injection was performed in horses sedated with xylazine hydrochloride (0.4mg/kg IV) and butorphanol (0.01mg/kg IV). Both MC joints were clipped and prepared aseptically, held in flexion and a 21 gauge, 1.5 inch needle was inserted into the dorsolateral aspect of the joint. Synovial fluid was collected from the joint prior to injection of 5×10^{11} viral genomes (vg) in 3ml of sterile DPBS or 3ml of sterile DPBS alone on day 0.

Clinical Response to Vector Injection

Prior to injection with the viral vector, horses were evaluated for lameness using the AAEP grading system (0-5),[26] range of motion of the carpi (0-4), joint effusion (0-4) and response to sustained joint flexion (0-4). The circumferences of both MC joints were measured using a flexible measuring tape. Lameness evaluations and joint circumference measurements were repeated at days 4, 7, 14, 28, and 56 post-injection.

Synovial fluid analysis

Whole blood from the jugular vein and synovial fluid from the MC joint of both forelimbs was collected immediately prior to viral vector injection and then at day 4, 7, 14, 28 and 56 post-injection. Cytological analysis of the synovial fluid was performed with total and differential leukocyte count performed by Coulter (Beckman Coulter Inc., Fullerton, CA). Smear evaluation was performed on Wrights-stained smears (Hema-Tek, Siemens, Germany). Synovial fluid was analyzed for IGF-1 concentration using ELISA (Quantikine, R&D Systems, Minneapolis, MN). Synovial fluid was also analyzed for cytokine production by fluorescent bead-based multiplex assays (IL-10, IFN-γ, IFN-α, sCD14, and CCL2).[27,28]

Synovial biopsy

Synovial tissue was collected from the dorsal joint pouch 56 days post-injection. Biopsies were performed with horses standing and sedated with detomidine (0.02mg/kg IV)

and butorphanol (0.01mg/kg IV). Following sterile preparation of the dorsal aspect of the MC joint, a scalpel was used to incise the skin and joint capsule and Ferris-Smith rongeurs were used to collect synovium. Samples were fixed in 4% paraformaldehyde or placed in a -80^{o}C freezer for RNA extraction. Synovial membrane was sectioned, stained with hematoxylin and eosin (H&E), and scored using a semi-quantitative scoring system.

IGF-I Gene expression analysis

Following pulverization using a mortar and pestle, tissues were mechanically homogenized using a battery operated pestle grinder and vortex, and then RNA was isolated from tissues using the PerfectPure RNA Tissue Kit (5 Prime, Gaithersburg, MD). Purity and concentration of the RNA was assessed by UV microspectrophotometry (NanoDrop 2000 Spectrophotometer, Thermo Scientific, Waltham, MA). Gene expression was quantified by real-time PCR using the Taqman One-Step RT-PCR technique (Absolute Quantitative PCR; ABI PRISM 7900 HT Sequence Detection System, Applied Biosystems, Foster City, CA). Primer Express Software Version 2.0 (Applied Biosystems, Foster City, CA) was used to design equine primers and dual-labeled fluorescent probes [6-carboxyfluorescein (FAM) as the 5' label (reporter dye) and 5-carboxymethylrhodamine (TAMRA) as the 3' label (quenching dye)]. Sequences for primers and probes were generated from GenBank (National Institutes of Health, Bethesda, MD). Primer sequences for 18S used as a housekeeping gene were as follows: forward, 5'-CGGCTTTGGTGACTCT AGATAACC-3' and reverse, 5'-CCATGG TAGGCACAGCGACTA-3' and for IGF-I were as follows: forward, 5'-TGTACTGCGCACCCCTCAA-3' and reverse, 5'-TTGTGTTCTTCAAATGTACTTCCTTC

TG-3. Gene expression of IGF-I was measured in duplicate for all samples. The total copy number of mRNA was determined using a validated standard curve and these values were normalized 18S.

In vitro transduction inhibition assay

Serum and synovial fluid samples collected at days 0, 28 and 56 were heat inactivated and analyzed by an in vitro neutralizing antibody assay [13]. Recombinant rAAV-LacZ (10^9vg/well) was diluted in serum-free DMEM and incubated with 2-fold serial dilutions (initial dilution, 1:5) of samples for 1 hour at 37^{o}C. The mixture was added to 96-well plates seeded with 1 x 10^5 Huh7 cells/well infected 2 hours prior with WT HAdV5 (50 viral particles/cell). Wells were incubated for 18-22 hours. Cells were then washed and lysed, and lysate developed with β-galactosidase assay kit for bioluminescence (Applied Biosystems, Foster City, CA). Neutralizing antibody titers were reported as highest sample dilution that inhibited rAAV-LacZ transduction (β-gal expression) by ≥50%, compared with mouse serum control.

Immunoglobulin isotyping

Immunoglobulin isotyping assays were performed as previously described with some modifications .[29,30] Enzyme-linked immunosorbent assay (ELISA) plates (Costar, Corning Inc., Corning, NY) were coated with rAAV2 or rAAV5 virions at 1μg/ml and incubated at 4^{o}C overnight. Plates were then incubated with blocking buffer (4% BSA/PBS) for 1 hour.

Plates were washed five times between all subsequent steps. Equine serum or synovial fluid samples were diluted 1:100 and incubated for 1 hour at 37^{o}C. Serum and synovial fluid samples with known high antibody titers from previous testing were serially diluted and used to establish a standard curve. The highest dilution was assigned an ELISA unit of 200 or 400 depending on the isotype being tested. Serum and synovial fluid samples with known negative titers were used as a negative reference sample. Plates were incubated for 1 hour with primary monoclonal antibodies including IgG1 (1:10), IgG4 (1:20), IgG5 (1:10), and IgM (1:20) (**Table 4.1**). A secondary peroxidase-conjugated anti-mouse IgG (H+L) antibody (Jackson ImmunoResearch, West Grove, PA) at 1:200,000 was added followed by tetramethyl benzidine substrate for chromagen development. Optical density was measured using a Tecan plate reader for absorbance at 450 nm.

Table 4.1. Primary monoclonal antibodies used for immunoglobulin isotyping

Antibody	Ig Subtype recognized
1-22	IgM
CVS 45	IgG1 (IgGa)
CVS 39	IgG4 (IgGb)
416-2	IgG5 (IgG(T))

T-cell intracellular cytokine assay

Whole blood was collected from horses 7 days prior to virus injection and at days 14 and 56 post-injection. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (Ficoll-Paque Plus; GE Healthcare, Piscataway, NJ). PBMCs (3 x 10^6) were cultured in 24-well plates in 1000μL of cell culture medium (Dulbecco's modified Eagles' medium [DMEM], 10% [vol/vol] fetal calf serum, 1% [vol/vol] nonessential amino acids, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 50 μg/ml gentamicin; all from Gibco, Invitrogen, Grand Island, NY). Stimulated cells were treated with phorbol myristate acetate (PMA)-ionomycin (Sigma, St. Louis, MO) as a positive control for T-cell activation, or with rAAV serotype 2 or 5. Non-stimulated cells were cultured in medium only. At 24 hours of culture, Brefeldin A (Sigma, St. Louis, MO) was added to all wells to inhibit cytokine excretion in preparation for intracellular cytokine staining. Following 48 hours of culture, cells were fixed, permeabilized, and stained for CD4 or CD8 and for IFN-γ expression by using directly labeled antibodies and isotype-matched controls as previously described [31]. A FACSCanto II flow cytometer (BD Biosciences, San Diego, CA) was used to analyze cells with fluorescence gates set according to isotype control staining.

PBMC cytokine secretion assay

PBMCs (6×10^5) were cultured in 96-well plates in 200 μl of culture medium as described above. Stimulated cells were treated with PMA-ionomycin or with rAAV serotype 2 or 5. Non-stimulated cells were cultured in medium only. Following 48 hours of culture at 37^{o}C supernatants were collected and analyzed for IL-10, IFN-γ, and IFN-α concentration using a fluorescent bead-based multiplex assay [27]. IL-6 concentrations were quantified using ELISA [32].

Statistics

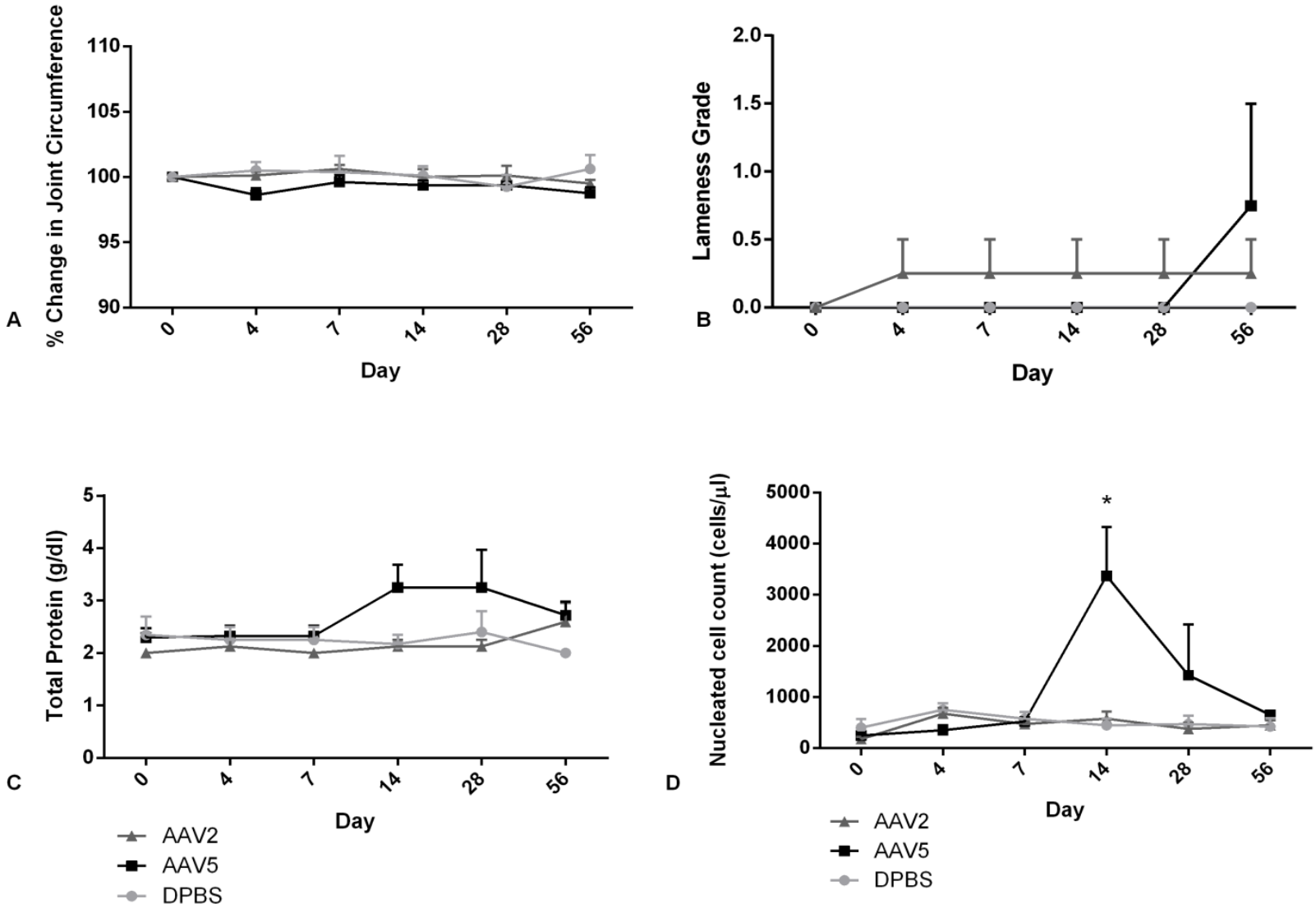
A Kruskal-Wallis test was used to compare differences between groups. A mixed effects model, with horse as a random effect, was constructed and least squared means was used to compare changes in synovial fluid composition and IGF-I concentration over time. Day was treated as a categorical variable to allow for the non-linear effect of time. Multiple comparisons for each interaction were made with a Tukey's *post hoc* test. Specific linear contrasts were fitted to the model where appropriate. A mixed effects model was also performed for analysis of associations between NAb titers, immunoglobulins, and IGF-I concentrations. Statistical analysis was performed using JMP. The level of significance was set at $p<0.05$.

Results

Joint response to vector injection

Injection of rAAV2-IGF-I or rAAV5-IGF-I did not induce greater articular inflammation compared to DPBS injected control joints at any time point. Lameness grades, % change in joint circumference, joint effusion, range of motion and response to sustained flexion were not different among any of the treatment groups at any time point (**Figure 4.1a,b**). Synovial total protein (TP) levels were slightly increased over baseline in rAAV5-IGF-I treated joints at days 14 (3.25 ± 0.44g/dl) and 28 (3.25 ± 0.72g/dl), however, these increases were not significant and returned to baseline by day 56 (**Figure 4.1c**). Total protein did not increase in rAAV2-IGF-I treated joints (**Figure 4.1c**). Synovial fluid total nucleated cell count (NCC) was significantly increased in rAAV5-IGF-I treated joints on day 14 (3375 ± 957.73 cells/μL; $p<0.0001$) only (**Figure 4.1d**). NCC in rAAV5-IGF-I treated joints decreased following this time point and were not significantly different than baseline on day 28 or 56 (**Figure 4.1d**). Differential cell counts revealed a trend in both rAAV2- and rAAV5-IGF-I treated joints, where the % of small mononuclear cells and polymorphonuclear cells (PMNs) decreased at day 4 and 7, while % of large mononuclear cells increased.

Figure 4.1. Joint response to middle carpal joint intra-articular administration of rAAV2-IGF-I, rAAV5-IGF-I, or saline. (a) % change in joint circumference, (b) lameness grade, (c) total protein concentration and (d) total nucleated cell count. Graphs illustrate mean values ± SEM averaged from 4 horses. * $p < 0.05$


A
% Change in Joint Circumference
110
105
100
95
90
0
4
7
14
28
56
Day
B
Lameness Grade
2.0
1.5
1.0
0.5
0.0
0
4
7
14
28
56
Day
C
Total Protein (g/dl)
5
4
3
2
1
0
0
4
7
14
28
56
Day
AAV2
AAV5
DPBS
D
Nucleated cell count (cells/µl)
5000
4000
3000
2000
1000
0
*
0
4
7
14
28
56
Day
AAV2
AAV5
DPBS

IGF-I synthesis and gene expression

IGF-I synthesis was quantified over time by measuring concentrations in the synovial fluid using ELISA. IGF-I concentrations in the synovial fluid from middle carpal joints of horses treated with rAAV5-IGF-I were significantly increased over joints treated with saline at days 14, 28 and 56 (**Figure 4.2a**). Synovial fluid IGF-I concentrations in rAAV2-IGF-I treated joints were significantly increased over saline treated joints at day 56 only. Additionally, synovial fluid IGF-I content from MC joints treated with rAAV5-IGF-I was significantly higher than rAAV2-IGF-I treated joints at days 14 and 28.

IGF-I gene expression in synovial membrane tissue collected at 56 days post-injection was quantified using qPCR. IGF-I mRNA expression was 25 times higher in synovial membrane tissue from joints treated with rAAV5-IGF-I and 10 times higher in synovial membrane tissue from joints treated with rAAV2-IGF-I, compared to joints treated with saline (**Figure 4.2b**). IGF-I expression in the synovial membrane from rAAV5-IGF-I treated joints was significantly increased over saline treatment ($p=0.03$) at 56 days post-transduction. Overall, IGF-I mRNA expression and synthesis was higher in rAAV5-IGF-I treated joints compared to rAAV2-IGF-I treated joints.

AAV2 and AAV5 capsid-specific $CD4^+$ and $CD8^+$ T-cell response

PBMCs were isolated from saline, rAAV2-IGF-I, and rAAV5-IGF-I injected horses at days -7 (prior to viral exposure), 14 and 56. T-cell responses in all 3 groups were assessed in

PBMCs re-stimulated with rAAV2 and rAAV5. Stimulated cells were stained with CD4, CD8, and IFN-γ antibodies and flow cytometry analyses were performed (**Figure 4.3**). After re-stimulation of PBMCs with rAAV2 or rAAV5, horses treated with either virus did not have increased IFN-γ^+ lymphocytes in either $CD4^+$ or $CD8^+$ populations indicating a lack of a cellular immune response (**Figure 4.4**). Supernatants collected from rAAV2 or rAAV5 stimulated PBMCs were also analyzed for production of IL-10, IFN-γ, IFN-α, and IL-6 and production characteristic of T-cell activation, viral infection or inflammation. Stimulation of cells from all 3 treatment groups, including saline and both AAV serotypes, did not induce significant amounts of IFN-γ (**Figure 4.5**), which correlates with the results obtained by flow cytometry analysis. Additionally, there were no significant changes in IL-10, IFN-α or IL-6 in any of the treatment groups.

Figure 4.2. (a) IGF-I concentration (ng/ml) in synovial fluid over time in joints injected with either rAAV2-IGF-I, rAAV5-IGF-I, or saline. Graphs illustrate mean values ± SEM averaged from 4 horses per group. * rAAV5 injected horses significantly increased over rAAV2 and saline injected horses, $p < 0.05$. ** rAAV5 and rAAV2 injected horses both significantly increased over saline injected horses, $p < 0.05$. (b) IGF-I mRNA expression in synovium 56 days after intra-articular injection of either rAAV2-IGF-I, rAAV5-IGF-I, or saline. * $p < 0.05$

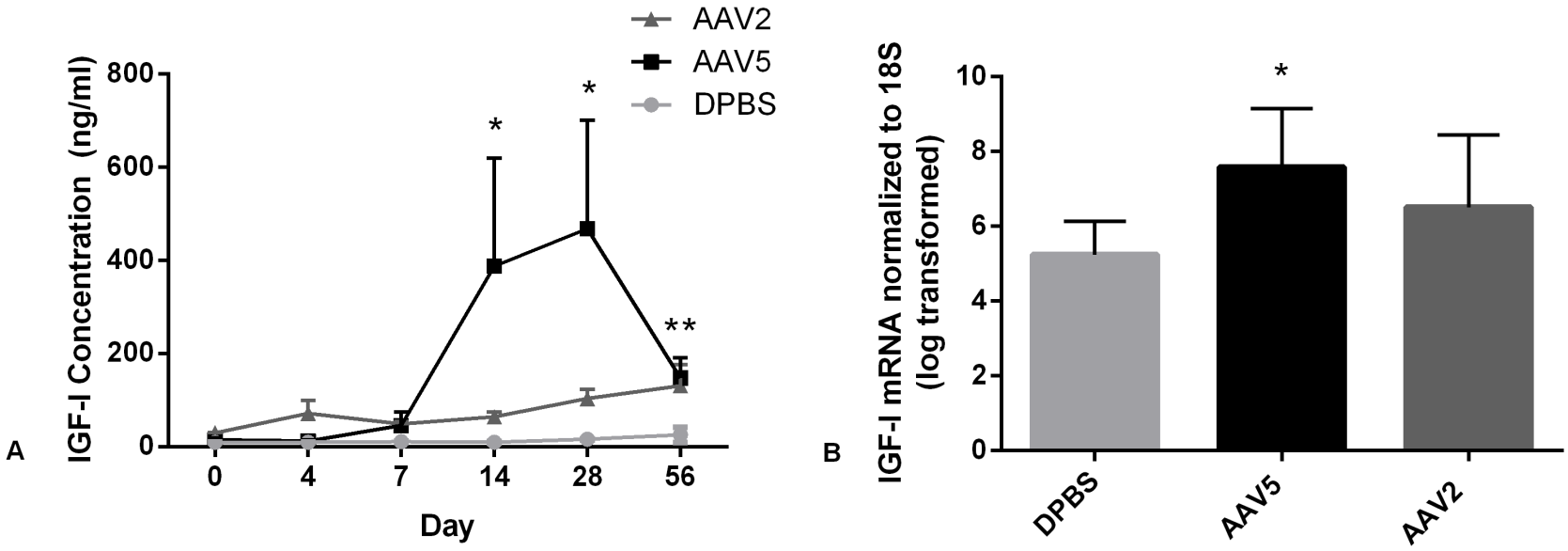
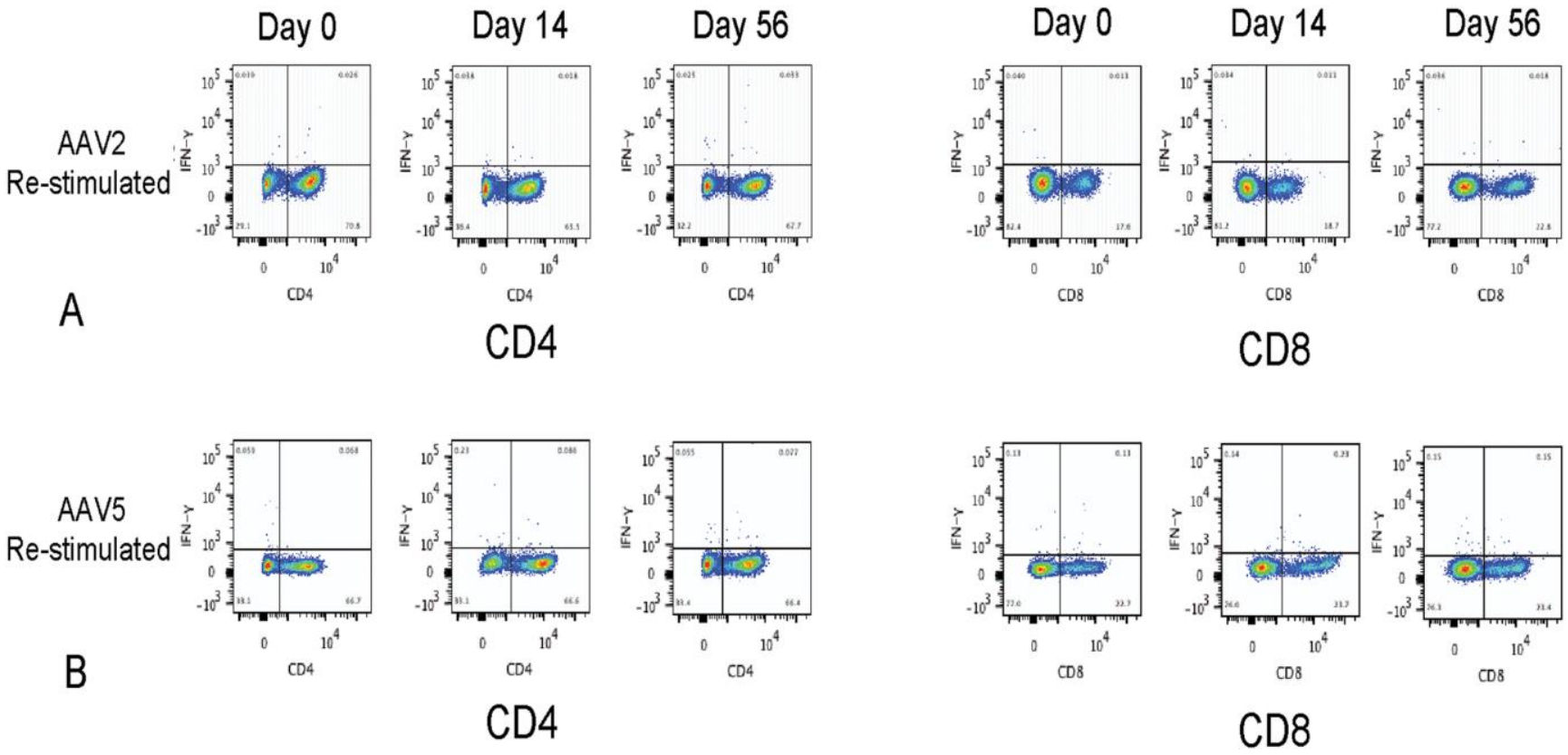

A
IGF-I Concentration (ng/ml)
800
600
400
200
0
0
4
7
14
28
56
Day
AAV2
AAV5
DPBS
*
*
**
B
IGF-I mRNA normalized to 18S
(log transformed)
10
8
6
4
2
0
*
DPBS
AAV5
AAV2

Figure 4.3. Representative flow cytometric plots of lymphocytes following T-cell re-stimulation assay with AAV2 or AAV5. PBMCs isolated from horses injected with saline, rAAV2-IGF-I, or rAAV5-IGF-I were collected at day 0 (prior to viral exposure), day 14, and day 56. Following re-stimulation, cells were stained for CD4, CD8 and intracellular IFN-γ. An isotype control was used to establish the quadrant gates for IFN-γ. (a) AAV2 re-stimulated PBMCs from rAAV2-IGF-I injected horses and (b) AAV5 re-stimulated PBMCs from rAAV5-IGF-I injected horses.


Day 0
Day 14
Day 56
AAV2
Re-stimulated
A
CD4
CD8
AAV5
Re-stimulated
B
IFN-γ
CD4
CD8

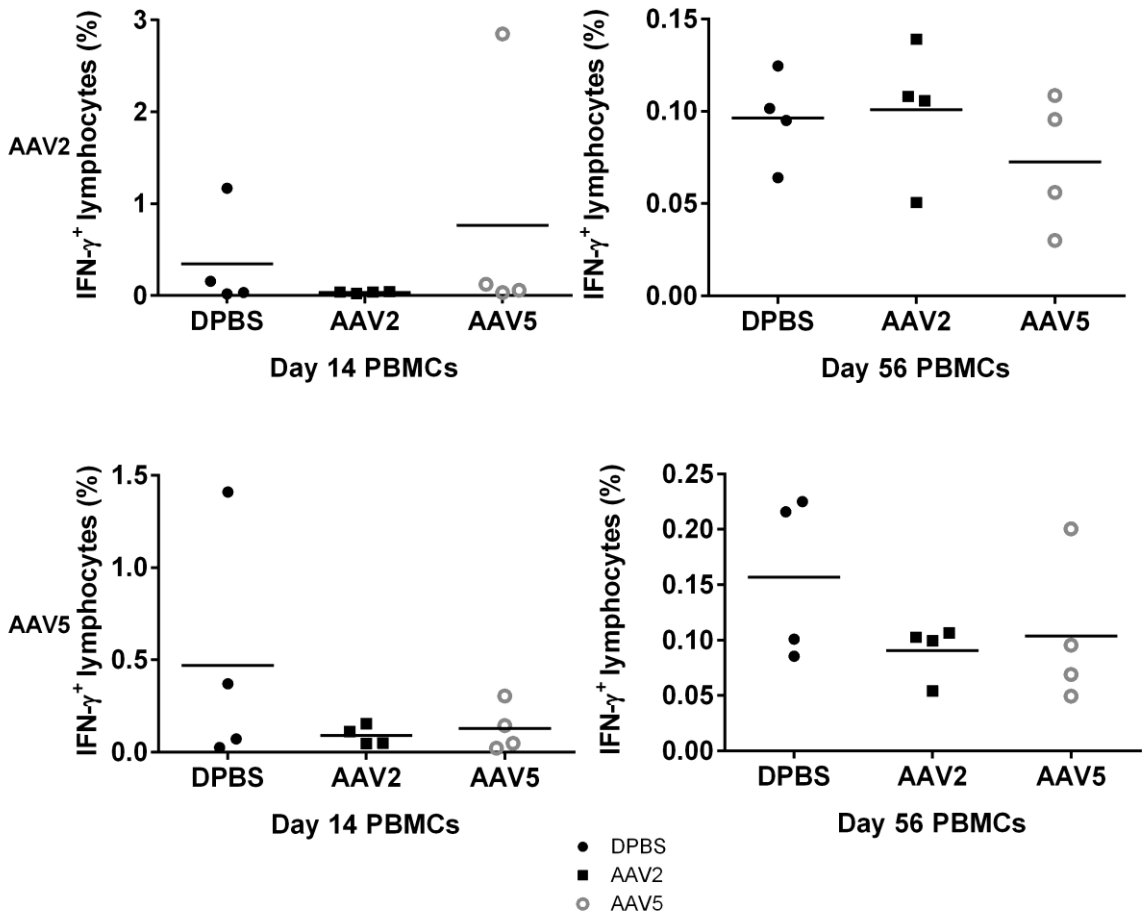


Figure 4.4. Total percentages of IFN-γ producing T cells from horses injected with DPBS, rAAV2-IGF-I, or rAAV5-IGF-I and re-stimulated with either rAAV2 or rAAV5 at day 14 and day 56.

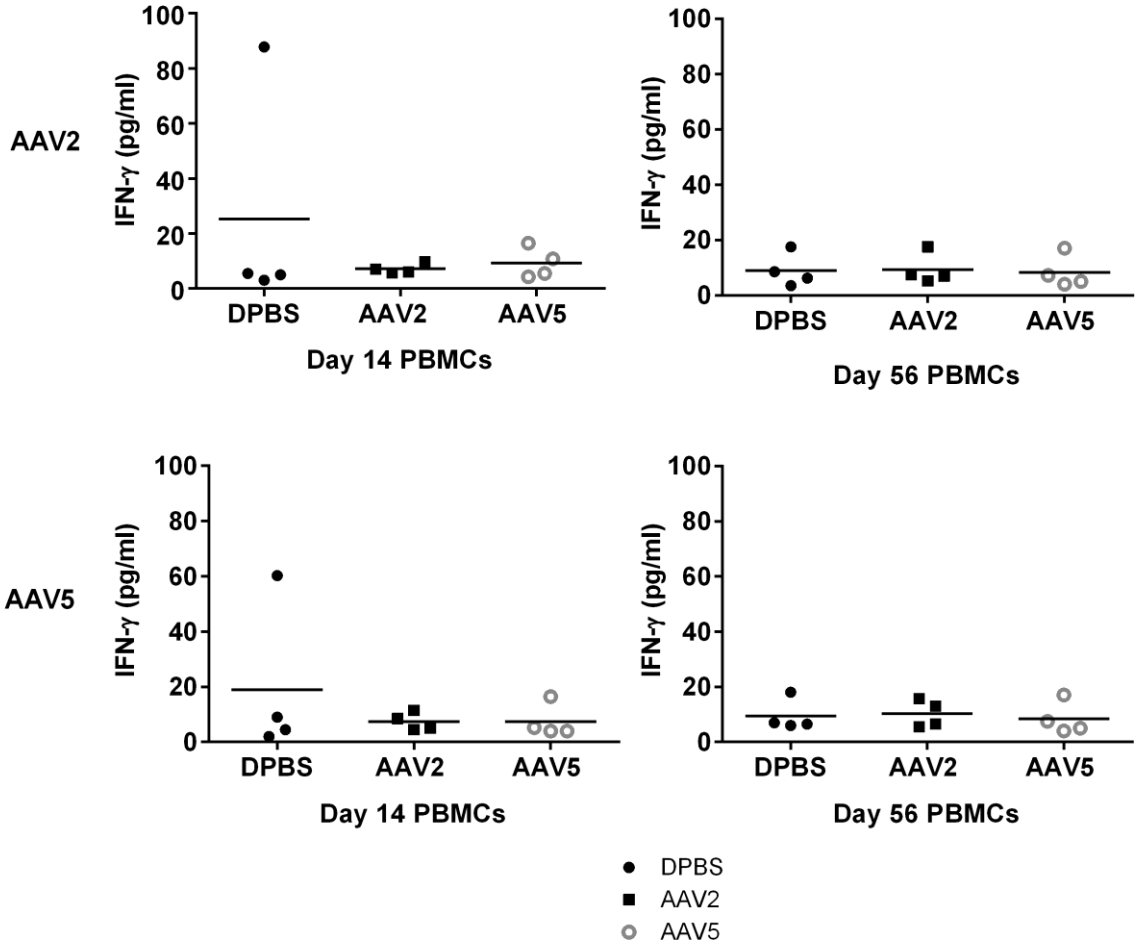
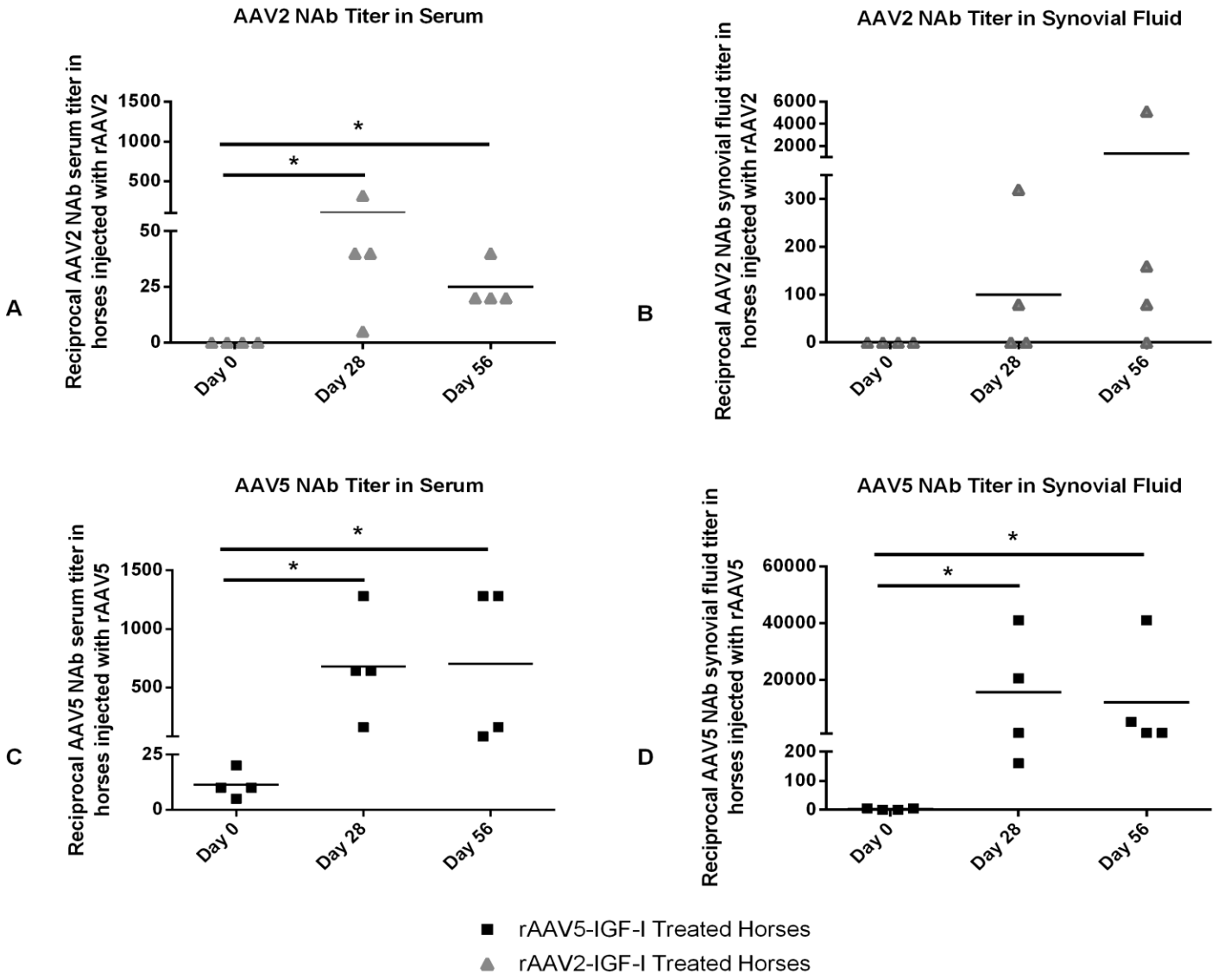


Figure 4.5. IFN-γ secretion (pg/ml) measured in the supernatants of rAAV2 or rAAV5 re-stimulated PBMCs of the 3 treatment groups at days 14 and 56.

Neutralizing antibody titers

Neutralizing antibody titers are shown in **Figure 4.6**. AAV2 NAb titers in serum and synovial fluid were measured in saline injected and rAAV2 injected horses, while AAV5 NAb titers in serum and synovial fluid were measured in saline injected and rAAV5 injected horses. All tested horses had pre-existing NAb titers to AAV5 in serum and 5/8 horses had NAb titers in synovial fluid. This is in contrast to AAV2 in which no tested horses had pre-existing NAb titers in either serum or synovial fluid. The NAb response at day 28 and 56 was more robust to AAV5 than AAV2 in both serum and synovial fluid (**Figure 4.6a-d**). AAV5 NAb titers were significantly increased in the serum at day 28 ($p=0.03$) and day 56 ($p=0.03$), and in the synovial fluid at day 28 ($p=0.03$) and day 56 ($p=0.03$) when compared to day 0 (**Figure 4.6a,b**). AAV2 NAb titers were only significantly increased in the serum at day 28 ($p=0.02$) and day 56 (0.02) (**Figure 4.6c**). The NAb titers in the contralateral saline injected joint in the rAAV5 treatment group were also increased at day 28 and 56. Contralateral joint titers were less than rAAV5 injected joint titers, however, there was a significant correlation between titers in the virally injected and saline injected joints ($r^2 = 0.74$; $p=0.003$). There was a significant correlation between serum and synovial fluid AAV5 NAb titers ($r^2 = 0.54$, $p=0.007$), however, there was not a significant correlation between serum and synovial fluid AAV2 NAb titers.

Figure 4.6. Reciprocal neutralizing antibody titers to AAV2 in serum (a) and synovial fluid (b) (n=4), and to AAV5 in serum (c) and synovial fluid (d) (n=4), from horses prior to exposure (day 0) and following IA rAAV2 or rAAV5 injection. A titer ≥ 1:5 was considered positive. * $p < 0.05$


A
AAV2 NAb Titer in Serum
Reciprocal AAV2 NAb serum titer in horses injected with rAAV2
1500
1000
500
50
25
0
*
*
Day 0
Day 28
Day 56
B
AAV2 NAb Titer in Synovial Fluid
Reciprocal AAV2 NAb synovial fluid titer in horses injected with rAAV2
6000
4000
2000
300
200
100
0
Day 0
Day 28
Day 56
C
AAV5 NAb Titer in Serum
Reciprocal AAV5 NAb serum titer in horses injected with rAAV5
1500
1000
500
25
0
*
*
Day 0
Day 28
Day 56
D
AAV5 NAb Titer in Synovial Fluid
Reciprocal AAV5 NAb synovial fluid titer in horses injected with rAAV5
60000
40000
20000
200
100
0
*
*
Day 0
Day 28
Day 56
rAAV5-IGF-I Treated Horses
rAAV2-IGF-I Treated Horses

Immunoglobulin isotypes

Prior to rAAV injection, 11/12 horses had detectable IgM against AAV2 in their serum while 10/12 horses had detectable IgM against AAV5 (**Figure 4.7**). All horses had detectable IgG5 to both AAV2 and AAV5 while 6/12 horses had detectable IgG4 to both serotypes. AAV2-specific IgM increased in the serum of horses injected with rAAV2-IGF-I at days 28 and 56 but these increases were not significant compared to the other treatment groups (**Fig 4.8a**). The synovial fluid IgM concentration had a trend towards increased concentrations in rAAV2-IGF-I injected horses at day 56 compared to saline and rAAV5-IGF-I injected horses ($p=0.06$). The synovial fluid IgG1 concentration was significantly increased in rAAV2-IGF-I injected horses at days 28 ($p=0.0041$) and 56 ($p=0.0054$) compared to saline and rAAV5-IGF-I injected horses (**Figure 4.8b**). The humoral response to AAV5 appeared to be more diverse as there were greater changes in immunogloblulin isotypes over time in the AAV5-specific ELISA compared to the AAV2-specific ELISA. AAV5-specific IgM was significantly increased in the serum of rAAV5-IGF-I treated horses at day 28 ($p=0.0154$) compared to the other treatment groups whereas AAV5-specific IgG1 was significantly increased at days 28 ($p=0.0092$) and 56 ($p=0.0054$) (**Figure 4.8c**). In the synovial fluid, IgM was significantly increased in rAAV5 treated horses at days 28 ($p=0.0054$) and 56 ($p=0.05$), IgG1 was significantly increased at days 28 ($p=0.0054$) and 56 ($p=0.0092$), and IgG4 was significantly increased at day 56 ($p=0.0261$) (**Figure 4.8d**). The amount of AAV2- or AAV5-specific IgG4 or IgG5 in the serum did not change significantly over time in any of the 3 treatment groups.

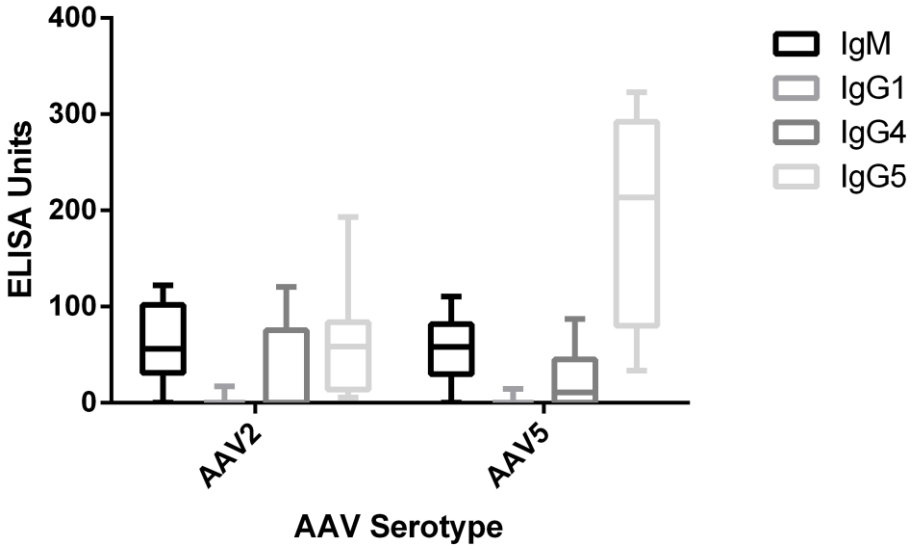
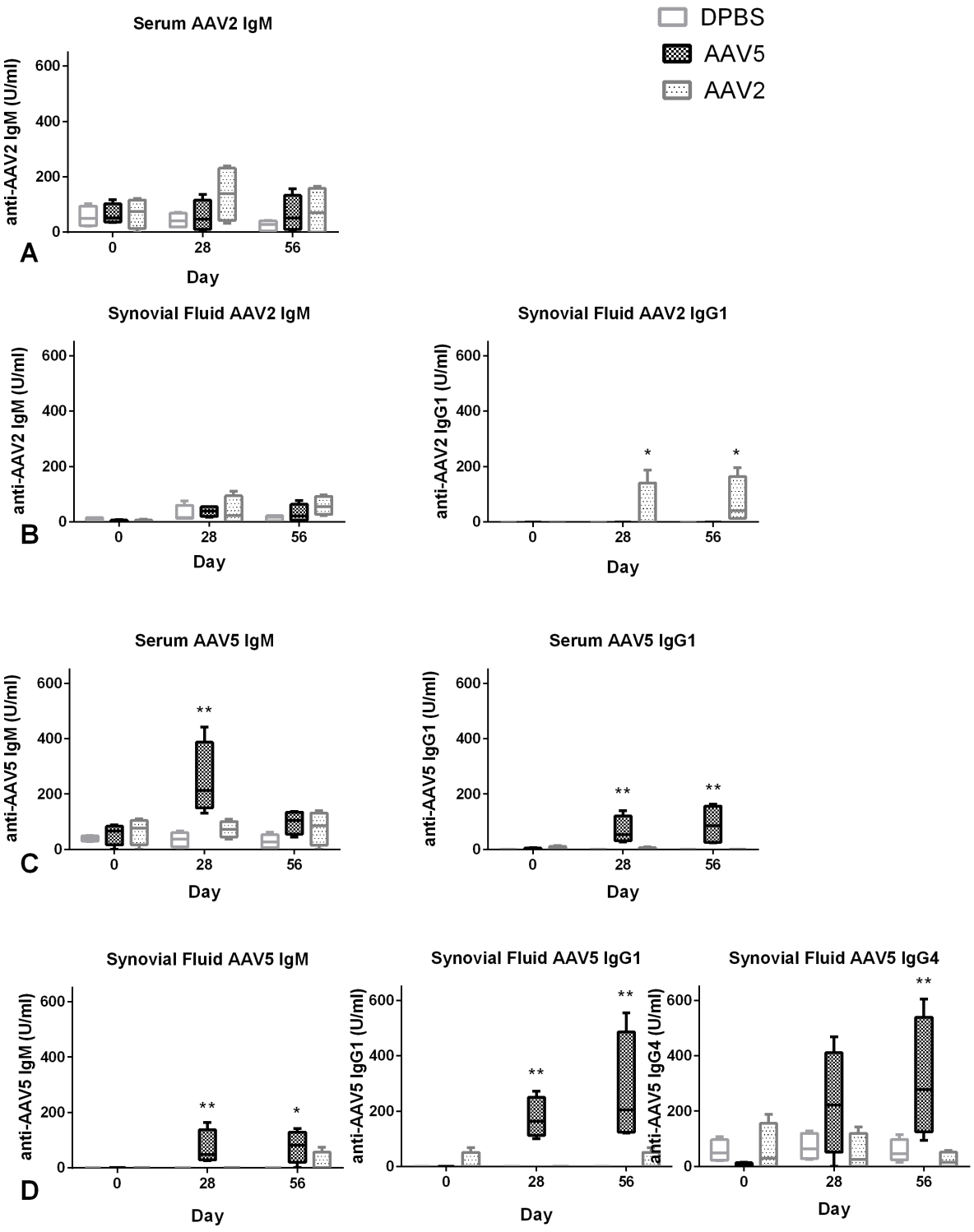


Figure 4.7. Pre-existing immunoglobulin isotypes to AAV2 and AAV5 in the serum of horses (n=12).

Figure 4.8. Immunoglobulin isotype ELISA results from saline, rAAV2, or rAAV5 injected horses. (a) rAAV2-specific isotypes in serum and (b) synovial fluid. (c) rAAV5-specific isotypes in serum and (d) synovial fluid. * $p < 0.05$; ** $p < 0.01$


DPBS
AAV5
AAV2
Serum AAV2 IgM
anti-AAV2 IgM (U/ml)
600
400
200
0
0
28
56
Day
A
Synovial Fluid AAV2 IgM
anti-AAV2 IgM (U/ml)
B
Synovial Fluid AAV2 IgG1
anti-AAV2 IgG1 (U/ml)
*
*
Serum AAV5 IgM
anti-AAV5 IgM (U/ml)
**
C
Serum AAV5 IgG1
anti-AAV5 IgG1 (U/ml)
**
**
Synovial Fluid AAV5 IgM
anti-AAV5 IgM (U/ml)
**
*
D
Synovial Fluid AAV5 IgG1
anti-AAV5 IgG1 (U/ml)
**
**
Synovial Fluid AAV5 IgG4
anti-AAV5 IgG4 (U/ml)
**

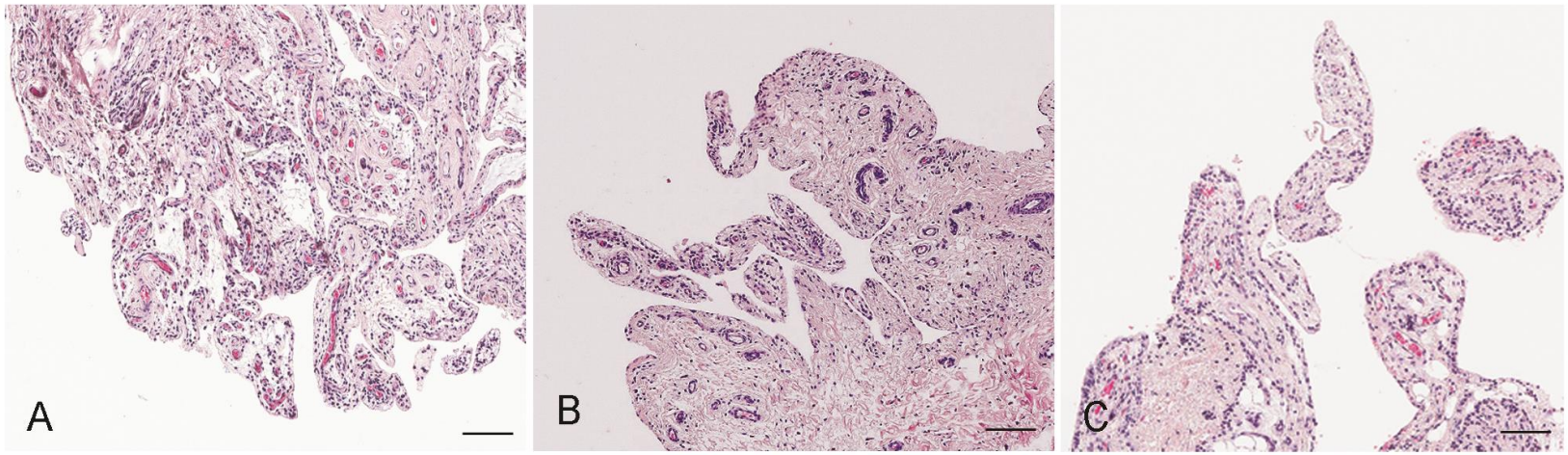
Serum and synovial fluid cytokine and chemokine concentrations

Serum collected from the 3 treatment groups was analyzed for IL-10, IFN- γ, IFN-α, sCD14, and CCL2 over time. There were no significant differences in IFN-γ, IFN-α, IL-10, or CCL2 concentrations in serum between the groups at any time point. Interestingly, the effect of day on sCD14 concentrations (p=0.001) but not treatment group was significant in the mixed effects model. sCD14 concentrations at day 28 (mean±SEM; 14452.6±803.8pg/ml) and day 56 (14495.9±762.7pg/ml) were significantly higher than sCD14 concentrations at day 0 (11982.9±942.5pg/ml), day 4 (12185.0±1036.0pg/ml) and day 7 (12455.4±617.6pg/ml). There was no detectable IL-10 or IFN-α in the synovial fluid of any horse at any time point. There were no significant differences in the amounts of IFN-γ, CCL2 or sCD14 in the synovial fluid between any of the treatment groups at any time point.

Synovial membrane histology

Synovial membrane biopsies collected from MC joints 56 days post-injection did not have significantly different scores between the treatment groups suggesting that direct injection of rAAV2 or rAAV5 does not cause a detectable inflammatory response in the synovial membrane as indicated by clubbing of villi, subintimal thickening, fibrosis, or inflammatory cell infiltration (**Figure 4.9**).

Figure 4.9. Photomicrographs of synovial membrane harvested from middle carpal joints 56 days following injection of (a) DPBS, (b) rAAV5-IGF-I, or (c) rAAV2-IGF-I. There were no significant differences in histological scores of synovial membrane biopsies obtained from the 3 treatment groups (d). Data in table presented as median (range) from 4 horses per group. Results analyzed using ANOVA.



Treatment Group	Villus Architecture	Villus Subintimal Fibrosis	Intimal Layer Thickening	Vasculature (# of vessels)	Inflammatory Cell Infiltrate (Perivascular Cuffing)	Total Score
DPBS	0 (0-1)	0 (0-1)	0	0 (0-1)	0	0.5 (0-2)
rAAV5-IGF-I	0 (0-1)	0	0	0 (0-1)	1 (1-3)	1.5 (1-4)
rAAV2-IGF-I	0	0	0	1 (0-1)	0 (0-1)	0 (0-2)

D

Correlation between humoral response and IGF-I concentrations in synovial fluid

Interestingly, in rAAV5 treated horses there was a significant positive correlation between IGF-I concentration and AAV5 NAb titers in the synovial fluid ($r^2 = 0.52$, $p=0.04$), suggesting that horses with higher synovial fluid NAb titers had higher transgene expression (**Figure 4.10**). No such relationship existed for rAAV2 treated horses. There were also no significant correlations between any specific immunoglobulin isotype in the serum or synovial fluid, and IGF-I expression in rAAV2- or rAAV5-IGF-I treated joints.

There was a significant positive correlation between AAV5 NAb titers in the serum and IgG1 in rAAV5 treated horses ($r^2 = 0.95$; $p<0.001$). No other significant correlations between AAV5 NAb serum or synovial fluid titers and immunoglobulin isotypes were observed. There was a weak correlation between AAV2 NAb titers in the serum and IgM in rAAV2 treated horses ($r^2 = 0.34$; $p=0.0394$). No other correlations between NAb titers and immunoglobulin isotypes were observed in rAAV2 treated horses.

Overall, rAAV5 incited a more diverse and robust humoral response compared to rAAV2 following IA injection. However, IGF-I concentrations in the synovial fluid and mRNA expression in the synovial tissue harvested at day 56 following rAAV5 injection were higher than in the rAAV2 treated joints.

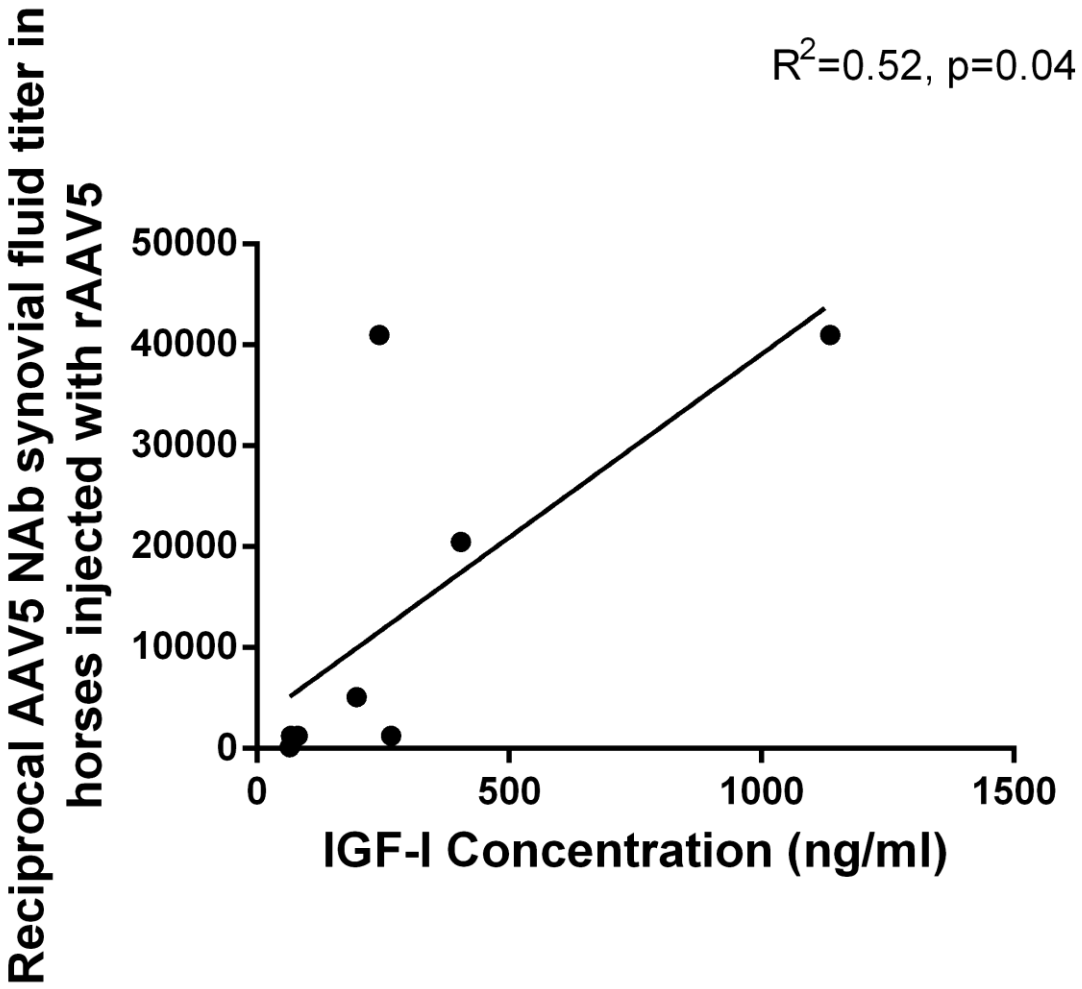


Figure 4.10. Relationship between synovial fluid IGF-I concentration and synovial fluid AAV5 NAb titers in horses injected with rAAV5-IGF-I. There was a significant, positive correlation between synovial fluid IGF-I concentration and NAb titer.

Discussion

rAAV2-IGF-I and rAAV5-IGF-I vectors administered intra-articularly to the middle carpal joints of horses were able to effectively transduce articular tissues with expression of the therapeutic transgene, IGF-I, throughout the 8 week study period. Importantly, injection of either vector did not cause any significant local inflammation as there were no changes in joint effusion, lameness scores, synovial membrane architecture, or synovial fluid nucleated cell counts and total protein concentrations. Although both serotypes successfully transduced host tissues, rAAV5-IGF-I showed superior transgene expression with higher synovial fluid concentrations of IGF-I and increased gene expression of IGF-I in the synovial tissue. This was in spite of all horses having pre-existing NAb titers to AAV5 and a more robust humoral immune response to rAAV5 than rAAV2, following injection. Transgene persistence was evident up to 56 days, with rAAV5-IGF-I expression peaking at 28 days and rAAV2-IGF-I peaking at 56 days. Synoviocytes are the predominant cell type transduced following IA injection as chondrocytes are largely sequestered in a thick extra-cellular matrix.[33,34] Although synoviocytes appear to be easily transduced, the half-life of these cells is less than 1 month[34] which can limit longevity of transgene expression in joints. Intra-articular transgene expression at 56 days suggests transduction of longer lasting cell types such as chondrocytes or fibroblasts in the joint capsule and supporting ligaments, as seen in murine joints injected with AAV.[35]

Initially, AAV vectors were deemed non-immunogenic based on early murine models.[36,37] Evidence of humoral and cell-mediated immune responses began to emerge as

different serotypes, hosts, and transgenes were investigated. Although the immune response to AAV appears to be significantly less than other vectors, such as Adenovirus, several studies have shown reduced transduction efficiency and longevity of transgene expression in animals with AAV titers as low as 1:4.[16,38] Few studies have evaluated the immune response, and its impact on transgene expression, following direct IA injection of AAV vectors. In order to further elucidate the immune response to IA AAV administration, we investigated the humoral and T-cell response in horses injected with rAAV2 or rAAV5 vectors.

Prior to treatment with either AAV serotype, all horses had low NAb titers to AAV5 while no horses had NAb titers to AAV2. The majority of horses had pre-existing detectable levels of rAAV2-specific IgM (92%) and AAV5-specific IgM (83%). One horse was IgM seronegative for both rAAV2 and rAAV5. All horses had pre-existing rAAV2- and rAAV5-specific IgG5. Pre-existing IgG4 against rAAV2 and rAAV5 was found in the 50% of horses; the same 6 horses were seropositive for both serotypes. Overall, there was a more diverse humoral response to rAAV5 injection than to rAAV2 with horses having significant increases in serum IgM and IgG1 levels and synovial fluid IgM, IgG1 and IgG4 levels. IgM showed an earlier peak at 28 days followed by decreasing levels at 56 days, while both IgG1 and IgG4 levels increased from day 28 to 56. The humoral response to rAAV2 appeared weaker with a small increase in serum IgM noted at day 28 and a significant increase in synovial fluid IgG1 at day 28 and 56. Although horses had no AAV2 NAb titers at day 0, detectable anti-AAV2 IgM, IgG4 and IgG5 were present in serum. This may be explained in part by the differing effector functions of immunoglobulin isotypes. For example, IgM is a strong activator of complement but has weak neutralizing abilities whereas neutralization is a major effector

function of most equine IgG subclasses, especially IgG1 and IgG4/7. In our study we also saw a strong correlation between AAV5 NAb titers in the serum and IgG1 in rAAV5 treated horses, which suggests that IgG1 may play a major role in neutralizing AAV. Interestingly, Chirmule *et al.* (1999)[39] also showed that 68% of AAV2-specific antibodies in humans were not neutralizing.

The humoral response to rAAV2 and rAAV5 in this group of horses was quite variable, with some horses being stronger responders than others. Several investigators have shown that the immunoglobulin subclass responses in humans are also widely variable with IgG1 responses being most prevalent but IgG2, IgG3 and IgG4 antibodies also being detected in most subjects.[40,41] It should be noted that the human and equine IgG nomenclature does not match and that isotypes with the same name are not necessarily functionally identical. However, in this study, equine IgG1 responses were also the most prevalent. Cross-reactivity of AAV antibodies has been shown to occur at low levels between some AAV serotypes,[42] however, in this study we did not see any significant cross-reactivity between the 2 serotypes used. From this study it is not possible to determine why horses had pre-existing antibodies to AAV2 and AAV5; two serotypes isolated from humans. It is possible that there is cross-reactivity with similar antigens that horses encounter naturally or that AAV has broad species tropism and can infect horses thereby stimulating a humoral response.

Despite the presence of pre-existing NAbs in all rAAV5 injected horses and the more robust humoral immune response to this serotype, probably due to an anamnestic response, rAAV5 appeared to be a more effective IA vector with higher levels of the transgene being

expressed throughout the study period. IGF-I concentration in the synovial fluid of rAAV5-IGF-I treated joints peaked at day 28, with a sharp decline noted at day 56. This may suggest loss of transduced cells due to an activated humoral immune system. Indeed, there were higher NAb titers in the rAAV5 injected joint compared to the contralateral saline injected joint which could support recruitment of NAbs to the transduced joint. Alternatively, induction of transgene specific $CD8^+$ T-cells could have decreased IGF-I expression, although this response was not examined in this study.

To the author's knowledge, no studies have examined the cell-mediated immune response to AAV in the horse. The horse serves as an important large animal model for human disease, especially diseases involving the joint. A greater understanding of the cell-mediated response is vital to development of clinically relevant AAV vectors for both equine and human patients. This study used several tools, including IFN-γ fluorescent bead-based assay and flow cytometry, to further elucidate the T-cell response to 2 commonly used AAV serotypes. Based on flow cytometry analysis of IFN-γ^+ T cells, re-stimulation of PBMCs with either serotype did not elicit an AAV-specific T-cell response. These findings were supported by lack of IFN-γ in the supernatants of re-stimulated cells. Memory CD8+ T-cell responses have been shown in humans,[18,21] although these responses are seemingly variable and dose-dependent. It is hypothesized that, following transduction and viral uncoating, some capsid remains in the cytosol and is processed by the proteasome prior to presentation on MHC I molecules. AAV capsid presentation by MHC I results in activation and expansion of capsid-specific $CD8^+$ memory T-cells with subsequent removal of transduced cells. $CD4^+$ T-cell responses to AAV seem to be less common in humans, possibly due to the poor transduction

efficiency of antigen presenting cells (APCs) by AAV.[43] The lack of AAV-specific CD8+ T-cells in this study may indicate that horses do not mount a significant cell-mediated response to AAV vectors. However, T-cell responses may have been limited due to the relatively small dose of vector (5×10^{11}vg/joint) and the IA nature of vector administration. The presence of rAAV2- and rAAV5-specific IgM responses and lack of CD8+ T-cell response, could suggest that AAV is acting as a T-cell independent antigen in the horse. Conversely, the immunoglobulin class-switching evident in many horses to various AAV-specific IgG isotypes is suggestive of a B-cell response that requires CD4+ T-cell help.

Supernatants from re-stimulated PBMCs, serum and synovial fluid were evaluated for changes in pro-inflammatory cytokines (IFN-γ, IFN-α, IL-6, IL-10). Both type I (IFN-α, IFN-β) and II interferons (IFN-γ) have been shown to increase in mice treated with AAV vectors; increases in IFN-α may be up-regulated by AAV following activation of TLR9 in response to intracellular viral genomes, while increases in IFN-γ appear to be associated with activated T-cells.[44] In this study, there was no evidence of an IFN-α or IFN-γ response in re-stimulated PBMCs, serum or synovial fluid at any time point. Additionally, no significant increases in IL-6 or IL-10 were seen following rAAV2 or rAAV5 administration. IL-10 has been shown to increase in human patients following administration of rAAV2,[41,45] while IL-6 transiently increased in mice following systemic injection of AAV.[44] The lack of significant changes in several pro-inflammatory and anti-inflammatory cytokines in these horses is encouraging, as it provides further evidence that these viral vectors are not inducing a significant innate or cell-mediated immune response.

Although serum or synovial fluid immunoglobulin concentrations did not limit transgene expression in this group of horses, it is possible that the humoral response observed following injection would limit effectiveness of re-administration of the vector, as a large dose of virus would again be presented to the primed adaptive immune system. Cottard *et al*. (2004)[46] demonstrated that the IgG antibodies in the synovial fluid are primarily responsible for limiting transduction efficiency; therefore, increased IgG concentrations following the primary AAV exposure could greatly complicate vector re-administration. If re-administration is required, a heterologous prime-boost strategy in which serologically distinct AAV serotypes are used will be crucial for successful treatment.

Limitations of this study include the small sample size in each treatment group especially given the variability in the humoral response observed. Increasing the number of horses per group would allow greater detection of differences. Additionally, it would have been more ideal to continue the study past 56 days given that IGF-I concentrations in the rAAV2-IGF-I administered joints peaked at day 56. A longer term study would allow a more comprehensive understanding of transgene expression and persistence by this serotype. Finally, re-administration of the vector would have shed light on the *in vivo* memory immune response and potential consequences of increasing AAV-specific IgG antibodies on transduction efficiency.

In conclusion, direct IA injection of rAAV2-IGF-I or rAAV5-IGF-I into the middle carpal joints of horses led to sustained, high transgene expression without significant local inflammation as determined through clinical parameters, synovial fluid composition and

synovial membrane histology. An AAV-specific T-cell response was not detectable in horses exposed to either serotype. Presence of pre-existing NAb to AAV5 did not appear to affect *in vivo* transduction and transgene expression. A humoral immune response was noted, especially in rAAV5 treated horses, although higher NAb titers in synovial fluid were actually correlated with increased IGF-I concentrations. This phenomenon could be indicative of a more robust tropism of rAAV5 for carpal joints compared to rAAV2, which would allow rAAV2 to leak out of the joint cavity and as a consequence minimize the local immune response and reduce the levels of IGF-I . Although, a significant cell-mediated response was not observed following IA injection in this group of horses, caution is needed when translating this information to human IA gene therapy as there appears to be significant differences in the nature of the immune response between species. Novel techniques such as capsid engineering and error-prone PCR to limit epitope presentation may represent feasible approaches to improving evasion of host immune responses.[47-49]

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CHAPTER 5

DISCUSSION

The overall goal of this dissertation research was to investigate AAV-mediated dual-axis gene therapy to improve cartilage repair and prevent osteoarthritis (OA). The first aim evaluated healing of full-thickness chondral defects implanted with autologous chondrocytes overexpressing the anabolic protein, IGF-I. Our second aim was to silence the catabolic cytokine, IL-1β, using a rAAV vector expressing a short hairpin RNA sequence, in chondrocytes cultured in an OA model. Due to the humoral immune response to AAV5 noted in the first aim, our third aim sought to investigate the humoral and cell-mediated immune response to direct intra-articular injection of rAAV2-IGF-I and rAAV5-IGF-I, and to evaluate the impact these responses had on expression of the transgene, IGF-I.

Improved healing in critically-sized (15mm diameter), full-thickness chondral defects was seen following implantation of autologous chondrocytes transduced *ex vivo* with an rAAV5-IGF-I vector compared to defects implanted with naïve chondrocytes or a cell-free fibrin graft. rAAV5-IGF-I repaired defects had better semi-quantitative healing scores at 8 weeks when second-look arthroscopy was performed. Improved healing scores persisted at 8 months post-implantation, when necropsy examination was performed. rAAV5-IGF-I implanted defects also had improved histologic appearance and increased collagen type II content at this time, which is encouraging as collagen type II is one of principal ECM components of hyaline cartilage and provides significant tensile strength to the articular surface.

The above results suggest that AAV-mediated overexpression of IGF-I improves healing capabilities of chondrocytes in the long-term. However, complete regeneration of native hyaline cartilage was not achieved and peripheral integration was not complete. Chondrocyte implantation and gene therapy has led to significant improvements in cartilage repair but repair tissue continues to be a mixture of fibrocartilage and hyaline cartilage, or "hyaline-like". When compared to native cartilage, repair tissue generally contains less collagen type II that is often disorganized, less proteoglycans indicative of a weaker ECM, and lack of complete peripheral integration. Taken together these shortcomings may indicate lack of durability and strength of repair tissue. It is possible that implanted chondrocytes are not surviving long enough in the graft to allow for reformation of hyaline cartilage. Chondrocyte survival could possibly be prolonged by overexpression of anti-apoptotic factors such as B-cell lymphoma (Bcl)-2 or through control of the catabolic cascade, which leads to NF-κB activation and possible cell death.

Activation of the catabolic cascade following injury to articular cartilage and during periods of homeostatic stress in chondrocytes (i.e., during culture and implantation) likely presents a significant barrier to cartilage repair, which cannot be solely overcome by overexpression of an anabolic protein. IL-1β is a key catabolic cytokine associated with degradation of the ECM which prompted us to investigate the effects of IL-1β gene silencing in chondrocytes cultured in an osteoarthritic model *in vitro*.

Post-transcriptional silencing of IL-1β was achieved in chondrocytes cultured under monolayer conditions using a rAAV2 vector expressing a targeting short hairpin RNA sequence. We chose to target IL-1β as it is the pivotal catabolic cytokine in damaged and/or inflamed cartilage. IL-1β is upregulated in activated chondrocytes and is responsible for upregulation of ECM degrading enzymes including MMPs and aggrecanases, inhibition of proteoglycan and collagen type II production, and is involved in the IGF-I hyporesponsiveness seen in osteoarthritic chondrocytes.[1] Effective IL-1β silencing following transduction with rAAV2-tdT-shIL-1β was confirmed by qPCR following stimulation of transduced chondrocytes with LPS. Several important downstream effects of IL-1β silencing were also noted, including significant downregulation of TNF-α and ADAMTS-5, both of which play major roles in degradation of the ECM. Additionally, we showed that IL-1β knockdown led to significantly decreased production of PGE_2 by stimulated chondrocytes. PGE_2 is a central inflammatory mediator involved in matrix degradation and the pain response associated with OA.[2] Similar downstream effects were not observed in expression of ADAMTS-4, MMP-3, or MMP-13, all of which remained unchanged following IL-1β knockdown.

Although there were several beneficial effects of IL-1β silencing, persistent suppression of key matrix proteins, including collagen type II and aggrecan, seen in chondrocytes transduced with rAAV2-tdT-shIL-1β were of concern. Additionally, increased expression of inflammatory mediators including RelA (p65) and IL-6 in transduced chondrocytes stimulated with LPS warrants further investigation, as these may be indicative of an intracellular immune response to AAV transduction, the presence of shRNA, or both.

Further experiments are required to evaluate the effect of AAV-mediated catabolic gene silencing in the equine chondrocyte to elucidate the intracellular response to this therapeutic approach. In addition, the use of 3-dimensional chondrocyte cultures, such as pellet, micromass or alginate cultures, or cartilage explant cultures may be more useful systems to evaluate cartilage specific effects of IL-1β knockdown. Due to the complex nature of RNA interference it is difficult to predict the *in vivo* effects of this technique using *in vitro* culture systems, thus an *in vivo* experiment may be required to fully investigate the benefits and drawbacks of IL-1β post-transcriptional silencing.

The first two aims clearly demonstrated that rAAV is an effective vector for transducing articular cells and expressing both a larger peptide (IGF-I) and short hairpin sequence for RNA interference; however, understanding the immune response to AAV is key to its success as a clinically useful vector. Innate and adaptive immune responses are capable of limiting vector effectiveness and transgene persistence, as well as creating significant safety concerns.

The third aim in this dissertation research, which evaluated the local and systemic immune response to intra-articular injection of a rAAV2 and rAAV5 vector, demonstrated that there is a moderate humoral response to these vectors, especially AAV5, but a minimal T-cell immune response in the horse. We also demonstrated the existence of pre-existing NAbs to AAV5, and a more robust and diverse humoral response to this serotype, which did not appear to significantly impact growth factor formation. Interestingly, all horses had pre-existing NAbs to AAV5 while no horses had pre-existing NAbs to AAV2; however, rAAV5-

IGF-I led to significantly higher levels of intra-articular IGF-I than rAAV2-IGF-I. Overall, the presence of pre-existing NAbs in serum and synovial fluid and a more robust humoral response did not appear to limit vector effectiveness. The lack of T-cell response observed in these horses, in comparison to humans, may be due to the route of administration (the joint is a relatively contained environment), use of low to moderate dose, or simply a difference in immune responses among species. Although the horse is an excellent model for joint disease and cartilage repair, extrapolation of this data to humans is difficult as AAV immune responses are extremely variable between species. Further investigations into the longevity of transgene expression and the effects of redosing joints with the same and alternative serotypes would be of great value. This would allow evaluation of the usefulness of rAAV vectors as direct intra-articular therapy modalities that could be used to maintain sustained therapeutic protein levels in the joint. The presence of pre-existing antibodies to AAV5 did not appear to limit transduction, as these joints had high levels of IGF-I; however, the significant increase in IgG1 and IgG4 observed following injection would likely limit transduction following redosing.

In summary, this dissertation research showed that AAV-mediated gene therapy can improve the repair capability of implanted chondrocytes, and alter key catabolic cytokines and degradative enzymes of cartilage, by influencing both the anabolic and catabolic cascade. AAV was capable of articular cell transduction and transgene expression *in vitro*, *ex vivo* and *in vivo*. Although AAV incited a humoral immune response following IA injection, a minimal inflammatory response in the joint and lack of a T-cell response are both encouraging findings.

Future investigations into the impact of concurrent growth factor overexpression and catabolic cytokine knockdown is warranted as it is logical to assume that a synergistic effect will occur. Together IGF-I and IL-1β are likely the two most important peptides in cartilage homeostasis with IGF-I promoting growth and repair, and IL-1β regulating degradation of the ECM.[3] Gene therapy targeting both peptides is an attractive strategy for managing the injured or degenerative joint as this may more effectively restore cartilage homeostasis. The anabolic and catabolic cascades of chondrocytes are intimately linked, and control of both using a combinatorial gene therapy approach will likely offer superior treatment outcomes.

References

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