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DISSERTATION

INTERLEUKIN-1 RECEPTOR ANTAGONIST DELIVERY THROUGH ADENOVIRAL MEDIATED GENE TRANSFER AS A TREATMENT FOR EQUINE JOINT DISEASE

SUBMITTED BY

DAVID D FRISBIE

DEPARTMENT OF CLINICAL SCIENCES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR

THE DEGREE OF DOCTOR OF PHILOSOPHY

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY DAVID D FRISBIE ENTITLED "INTERLEUKIN-1 RECEPTOR ANTAGONIST DELIVERY THROUGH ADENOVIRAL MEDIATED GENE TRANSFER AS A TREATMENT FOR EQUINE JOINT DISEASE" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

COMMITTEE ON GRADUATE WORK

Advisor

Department Head

ABSTRACT OF DISSERTATION

INTERLEUKIN-1 RECEPTOR ANTAGONIST DELIVERY THROUGH ADENOVIRAL MEDIATED GENE TRANSFER AS A TREATMENT FOR EQUINE JOINT DISEASE

The interleukin-1 (IL-1) system is a small group of cytokines composed of two agonist proteins and a receptor antagonist (IL-1Ra). The action of the IL-1 system is mediated through binding of these ligands to the IL-1 receptors. Binding of the IL-1Ra protein to IL-1 receptors has not been associated with biological activity and serves to block the binding of the other ligands. The IL-1 system has a wide range of biological activities centrally involved in the genesis and maintenance of inflammatory responses. More specifically, it has been centrally linked to mediation of joint pathology in most species. This project was conducted to assess both ability to deliver a transgene to the joints of horses, and the specific effects overproduction of IL-1Ra using gene transfer would have on an experimental model of joint disease in the exercising horse.

Using the published gene sequence for equine IL-1Ra, an adenoviral vector (Ad-EqIL-1Ra) was constructed that was capable of equine IL-1Ra transgene expression. This vector was tested *in vitro* to ensure its ability to both transduce equine synoviocytes without cytotoxic effects and produce a biologically active IL-1Ra molecule. A dose titration of the vector was conducted *in vivo,* in the equine metacarpal and intercarpal joints, to ascertain the concentration of vector that produced the highest transgene expression for the longest period of time without significant deleterious effects. The

result of this *in vivo* work suggested a vector concentration of 20 X 10^{10} particles/joint met the defined criterion.

Using an experimental model of equine joint disease, the ability of intra-articular (IA) administration of Ad-EqIL-1Ra to effect osteochondral fragment induced joint pathology was evaluated. This work demonstrated an approximately 28 day effective upregulation of IL-1Ra expression through IA administration of Ad-EqIL-1Ra. This increased level of IL-1Ra was associated with significant improvement in clinical parameters of pain and disease activity, as well as beneficial effects in histologic parameters measured from synovial membrane and articular cartilage. This study provided proof of principle that gene therapy, using a potentially anti-arthritic gene sequences, is possible and efficacious in the horse and will hopefully serve as a cornerstone in future equine therapeutics.

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PREFACE

The study of joint disease is an ever-evolving field, which despite great strides in defining the pathology and treatment of the disease processes, has generated more questions than answers. These questions along with the quest for complete understanding are in part what drives the continued research.

Researchers in equine joint disease are a small subset in a vast scientific community. However, through collaborative efforts, state of the art research is both brought to and generated by this small subset of people. The work presented here is an effort to bring a novel treatment modality to equine joint therapeutics, one that is hopefully both flexible and can withstand the test of time. Through the development of this technology, we hope to not only provide therapeutic benefits but also to gain a better understanding of specific equine joint disease entities.

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CHAPTER 1

1 Introduction

1.1 Purpose

The purpose of the following research was to test the utility of gene therapy as a novel treatment for equine joint disease. The proposed research would include: (1) the creation of a vector possessing anti-arthritic potential; (2) testing the utility of this vector and its ability to produce a biologically active transgene product *in vitro*; (3) *in vivo* dose titration of the vector in joints of normal horses; (4) *in vivo* testing of this application in an osteochondral fragment exercise model.

1.2 Goals

The short-term goals to be completed were: (1) To determine if gene transfer in the equine joint was possible and (2) To test the anti-arthritic potential of interleukin-1 receptor antagonist protein (IL-1Ra) through the use of gene transfer. The long-term goal was to develop a methodology for administering anti-arthritic therapeutics to the equine joint or other musculoskeletal tissues in a manner flexible enough to withstand the test of time.

1.3 Statement of Hypothesis

The hypothesis to be tested states: Using gene transfer to deliver IL-1Ra to the synovial cavity will significantly reduce damage to joint tissues induced by a traumatic and inflammatory model of osteoarthritis (OA).

1.4 Specific Aims

1.4.1 In Vitro (Chapter 2)

(1) To establish the transduction efficiency of equine synoviocytes using characterized vectors carrying marker genes.

(2) To establish the detectable level and biologic activity of equine IL-1Ra produced after *in vitro* transduction of equine synoviocytes using an adenoviral vector carrying the equine IL-1Ra gene (Ad-EqIL-1Ra).

1.4.2 In Vivo – Normal Horses (Chapter 2)

(1) To establish the *in vivo* concentration and duration of expression of equine IL-1Ra for five different concentrations of the Ad-EqIL-1Ra vector.

1.4.3 In Vivo – OA Model (Chapter 3)

(1) To measure the therapeutic effects of equine IL-1Ra on a traumatic and inflammatory model of OA in exercising horses.

1.5 Background, Significance & Rationale

1.5.1 Importance of the Disease

Diseases of the muscoloskeletal system are among the most common equine and human afflictions. [1-3] In a recent national survey, "injuries/wounds/trauma and/or

leg/hoof problems accounted for the greatest number of days of lost use and for the greatest cost for more operations in 1997 than any other health condition".[4] Most of these problems related to the musculoskeletal system and were probably joint related. In another survey of equine patients, 19% had lameness as their presenting complaint and 42% of these lame equine patients had lameness directly related to joint disease.[1] Specifically OA has been cited as the most economically important musculoskeletal disease in both athletic and pleasure horses.[5-7] Likewise in humans, OA is the most common arthritic condition and affects 20.7 million (12.1%) of American adults. Furthermore, after chronic heart disease, it is the second-leading cause of long-term, paid disability in the US.[3] These statistics emphasize the importance of musculoskeletal diseases in horses and humans, therefore supporting the need for continued research and improvements in current therapeutics.

1.5.2 Conventional Therapeutics in Joint Disease

Numerous medical treatments have been used extensively in the management of joint diseases. To date, these treatments have been directed toward lowering and then maintaining a decreased degree of inflammation within damaged joints.[8] Little attention has been focused on therapeutic agents that actually protect the joint tissues (disease modifying agents). Non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids have been the primary mode of anti-inflammatory therapy. Although NSAIDs often provide symptomatic relief, little protection is afforded to the articular cartilage, nor do the drugs modify the underlying disease process. NSAIDs are also associated with a high incidence of undesirable side effects,[8, 9] making their long term use in joint disease unattractive. Corticosteroids are also commonly used to treat joint disease, and they are potent mediators of reducing pain and inflammation. However, corticosteroid use has been associated with untoward effects on articular cartilage,

including impaired chondrocyte activity, decreased glycosaminoglycan and proteoglycan content, and decreased cartilage elasticity. [10-13] These effects have led to claims of negative effects associated with corticosteroid use. [14-16] To date, no one therapeutic agent has effectively, and without side-effects, eliminated the progression of joint disease.

Therapeutic intervention in joint disease is hindered in part by the inability to target therapeutic agents directly to the joint. Traditionally, oral, intravenous, and intramuscular routes have delivered therapeutic agents. These routes of administration can be relatively ineffective, since small molecules enter the joint space by passive diffusion, and large molecules (such as proteins) are generally excluded from the joint space.[17] Although intraarticular (IA) administration by-passes these limitations, the half-life of most agents directly administered into the joint space is short, [18] and frequent IA injections are needed to sustain biologic activity for prolonged treatment of chronic diseases. In addition, many drugs cannot be used by direct IA injection. In an attempt to circumvent some of these limitations, gene transfer is being investigated to deliver proteins to the joint space.

Gene transfer provides an excellent potential alternative, whereby a single IA injection can result in the local production of specific proteins within a diseased joint(s) for prolonged periods of time.[19] As our understanding of joint disease progresses, gene transfer, as a therapeutic modality, requires further investigation especially given the flexibility of this administration system to express new gene sequences as they and their biological roles become understood.

1.5.3 Perspectives in gene therapy

Gene therapy relies on three different key components: 1) A gene sequence of interest. In most cases for treatment of joint disease, the gene sequence of interest is

chosen based on the known biological actions of the protein it codes for. 2) A methodology of transferring the vector to the area of interest. In joint disease, this is often to a specific joint but in cases of rheumatoid or multiple joint arthropathies, systemic treatment may be advantageous. 3) A vector, that provides a mechanism by which the gene of interest is transported to the target tissue, allows or facilitates the entry of the gene of interest to the cell's nucleus and in most cases drives the transcription / translation of the gene of interest. The selection of the gene of interest (IL-1Ra in these studies) is covered within a subsequent section entitled **Possible anti-arthritic agents** while the latter 2 key components will be covered in this section.

1.5.3.1 Transfer methodology

Many different therapeutic strategies in gene transfer are possible at this time, and new methodologies to transfer genes are being developed every day.[20, 21] One needs to consider the strategy best suited for joint disease. Systemic and local therapies both have advantages and disadvantages. Systemic therapy would allow secreted gene products access to the systemic circulation and the gene product to be delivered to all joints. However, if the gene product is a protein, joint access may still be limited. Preliminary experiments in rodents have shown that large amounts of anti-arthritic proteins (specifically IL-1Ra) can be produced systemically, but do not reproducibly protect subjects from induced arthritis.[22] In contrast, IA expression (local therapy) of the same protein appears to confer protective effects.[22] Local therapy dictates multiple injections if numerous joints are involved, much like the current practice of IA corticosteroid therapy. However, the advantages of local versus systemic therapy are the likelihood that unwanted side effects will be minimal, because the gene products will be synthesized IA and the highest concentrations will be seen in the affected area. Also, the non-target organs will receive less exposure. The synovium as a target tissue

is also attractive because of its large surface area and its direct contact with the joint space. While the articular cartilage is another available target tissue within the joint, lack of vector penetration through the extensive extracellular matrix has been sited as a cause of poor transduction efficiencies, thus limiting the articular cartilage as a target for gene transfer to date. Given good transduction efficiencies and easy access to the synovial membrane, the low number of joints typically affected in equine joint disease individual IA joint treatment transferring vectors to the synovial membrane remains logical and is the method chosen for the following studies.

Further considering local gene therapy, two methods of transferring genes to the joints of interest can be utilized, *ex vivo* and *in vivo*. *Ex vivo* gene transfer refers to the harvest of cells, the genetic modification *in vitro* followed by the grafting of cells back into the patient. While *in vivo* gene transfer refers to the direct application of the vector to the target tissues. Both methodologies will be explored in the following paragraphs.

Ex vivo delivery systems have been extensively researched in rabbits and rodents and have been shown to efficiently transfer genes to the synovial lining using retroviral vectors, one of the most common vectors used in *ex vivo* delivery systems.[22-25] When applying this methodology to joint disease, synovial cells are surgically removed from the subject, used as a source of synoviocytes that are grown in culture and then infected by a vector encoding a gene of interest. These cells are then returned to the subject's joint space via IA injection. To date, this method has shown production of marker proteins as well as several nanograms of biologically active IL-1Ra in rabbits and rodents.[23-25] IL-1Ra levels were detected in joints containing transduced synoviocytes for approximately 5 weeks, although levels of IL-1Ra were rapidly declining at this time period.[24] Gene transfer using an *ex vivo* approach alleviates viral particles being introduced into the joint (limiting sensitization to the vector) and allows all genetic manipulations to occur outside the body (safety testing can occur prior to re-introduction

of cells back to the host). Historically, *ex vivo* systems have been approved for gene therapy in the fields of ADA-deficiencies, immunotherapy for AIDS, cancer, and rheumatoid arthritis,[26-28] providing evidence for their safety and efficacy. A major limitation of this methodology lies in the time consuming nature of harvesting, manipulating and re-implanting the transduced cells in addition to the cumbersome clinical applicability of this methodology. Studies published on a form of cancer therapy that entails many of the same manipulations required in *ex vivo* gene transfer for joint disease, has quoted an average cost of $20,000 per patient.[29] This is a serious hurdle to the commercialization of *ex vivo*-delivered gene therapies in both the horse and humans. For this and the previously listed reasons, *in vivo* gene transfer has been the focus of many recent gene therapy protocols and is the methodology utilized in these studies.

Typically when using *in vivo* gene transfer for the treatment of joint disease, the vector is directly injected into the joint(s) of interest, making this technique very clinically applicable and alleviating the time consuming and economic concerns associated with *ex vivo* gene transfer. Because *in vivo* delivery systems involve the direct introduction of vectors into the body, the control of these vectors is less stringent than in *ex vivo* gene transfer systems, although to date there have been no significant side effects reported.[30-32] Furthermore, most of the corporate research programs in the pre-clinical stages utilize *in vivo* gene transfer methods, and this trend is predicted to continue.[32] Specific examples of vectors utilized for *in vivo* methods are outlined in more detail in the following sections.

1.5.3.2 Vectors

An ideal vector should have the following three properties: (1) ability to integrate and sustain expression of the transgene; (2) no immunological consequences attributed

to the vector; and (3) no adverse pathological consequences to the recipient. As with most technologies, the ideal system is an ever-evolving pursuit; vector technology is still evolving and has been referred to as the rate-limiting step for successful gene therapy.[33]

Classification of vectors are generally either non-viral (synthetic) or viral. Non-viral vectors typically refer to synthetic molecules that facilitate the uptake of DNA into cells by condensing the DNA with lipids, peptides, proteins, inactivated virus particles, crystals of calcium phosphate, or coated microprojectiles. Viral vectors are viruses from which the viral genes have been removed to allow insertion of the therapeutic gene(s), and the viral vector has usually been rendered incapable of replicative spread. As a general rule, viral vectors give much higher efficiencies of gene delivery than non-viral vectors. Although progress in transfection efficiencies utilizing non-viral vectors is ongoing at the time of these studies, this technology has not developed to a point justifying further consideration. This is compared to many well-characterized viruses that have been explored for use as engineered vectors. To date, retroviral and adenoviral vectors have proven the most useful and will be considered in the following sections.[30, 32, 34]

1.5.3.2.1 Retroviral vectors

Retroviral vectors integrate their DNA (including a gene sequence of interest) into the chromosomal DNA of the target cells, ensuring gene transfer to the target cell progeny. In theory, this would provide long term transgene expression (protein expression from the gene of interest). However, the integration of DNA coding for a gene of interest still does not guarantee mRNA or protein expression, or even duration of transgene expression. For example, in cell culture, retrovirally transduced cells have shown DNA integration and long term expression of both marker and therapeutic

proteins but short term expression of these same proteins were observed *in vivo*.[24] The mechanism by which retroviral transgene expression is lost is not thoroughly understood, although loss of cells carrying the gene of interest through immunogenic pathways has been proposed.[24] This response may be triggered secondary to viral infection or viral transgene protein expression, especially if the transgene is that of another species. In some cases, however, inactivation of transgene expression has been shown to limit protein production even when the transgene DNA is still detectable.[24] Further limitation of the retroviral vector is its ability to only transduce actively dividing cells, hence its almost exclusive utilization for *ex vivo* gene transfer. However, this property also makes it useful in certain situations. For example, if the target population of cells were neuronal (non-dividing), and the retroviral vector carried a gene causing cell death, this vector could be quite useful in a gene transfer protocol for rapidly dividing neoplastic cells invading brain tissues. Although *in vivo* use of retroviral vectors has been explored for use in treatment of joint diseases, active synovitis is a prerequisite for effective synoviocyte transduction.[35] Without an accurate method of predicting synovial tissue mitotic rates, the ability to control synoviocyte transduction and protein expression using this form of gene transfer may be limited.

The inability to transduce non-proliferating cells has been overcome by a sub-family of retrovirus, the lentivirus. Researchers have shown that, at least in the case of the human lentivirus HIV, the viral DNA is actively transported to the nucleus through the nucleopores instead of relying on disintegration of the nuclear membrane during cell division for retroviral DNA entry to the nucleus.[36] From this work, lentiviral vectors have been developed with the goal of maintaining the ability of the virus to infect non-dividing cells while completely disabling its replicative and pathogenetic potential.[37] Initial work with lentiviral vectors has shown promising results, with animals analyzed 9 months after a single injection of the vector, the longest time tested thus far, showing no

decrease in the average level of transgene expression and no sign of tissue pathology or immune reaction.[38] The production of lentiviral vectors poses serious challenges because of the toxic nature of some of the components used in the system. In particular, one component causes the cells making the vectors to arrest in cell cycle phase-G2 thus prohibiting the propagation of the cell lines. Also, proteins associated with the envelope of the vector are toxic to the cells in which they are produced, further limiting the use of lentiviral vectors. Currently a number of approaches are being utilized to overcome some of these hurdles that may bring the lentiviral vectors to the forefront of gene transfer technology in the future.[39]

1.5.3.2.2 Adenoviral vectors

The use of adenoviral vectors in current gene transfer protocols has become increasingly prevalent[32] and some have predicted their use may exceed that of retroviral vectors in the future.[29, 32, 40] Properties making adenoviral vectors superior to retroviral vectors are also those which make retroviral vectors unfavorable, such as the ability to infect a wide host of cell types both dividing and non-dividing cells. Conversely, some of the unfavorable properties of adenoviral vectors are the advantageous properties of retroviral vectors, such as the ability to integrate in the host cell genome, lack of immunogenicity, and ability to reliably create replication incompetent vectors. Because an adenoviral vector was utilized in these studies, the history of this technology will be explored in the following sections in more detail as compared to other vector systems.

1.5.3.2.2.1 Molecular biology of adenovirus

Wild-type adenoviruses are responsible for a variety of clinical manifestations in humans such as respiratory infection, conjunctivitis, hemorrhagic cystitis and

gastroenteritis. They have also been identified in avian and mammalian species since their discovery in 1953.[41, 42] Based on their immunological, biological, and biochemical characteristics, there are 49 adenovirus serotypes (Ad1 – Ad49), and their hemagglutination properties define six distinguishable subgroups (subgroup A to subgroup F).[43] The best characterized types are Ad type 2 (Ad2) and Ad type 5 (Ad5) of the subgroup C. Both have been sequenced completely and are identical in 95% of the genome.[44, 45] Adenoviral vectors have been extensively utilized in mammalian cell expression systems prior to their initial use for human gene therapy, on April 17, 1993.[33]

Adenoviruses are non-enveloped double-stranded linear DNA viruses with a 30-40 Kbp genome. The genomic DNA is contained within an icosahedral protein coat or capsid (Figure 1). The protein coat comprises 240 hexons and 12 pentons forming 252 capsomers. Each penton is composed of a penton base and a fiber. The fiber is made up of a tail, rod-like shaft and a terminal globular structure called the knob (Figure 1). The genome is flanked by short (about 100-140 bp) inverted terminal repeat (ITR) sequences, each containing an origin of DNA replication. A *cis*-acting packaging domain, required for encapsidation of the genome, is located near the ITR at the "left" (relative to the conventional map of Ad; Figure 2). The genome can be divided into regions that are expressed, for the most part, either early or late after infection of the host cell, with the onset of DNA replication delineating the boundary of the two phases. The host RNA polymerase II transcribes most of the viral genome. The early region (designated "E" genes) transcription units are early region 1A (E1A), E1B, E2, E3, and E4. The gene-encoding protein IX (pIX), which is colinear with E1B but utilizes a different promoter, is expressed at an intermediate time, as is the PIVa2 gene. Most late transcripts initiate from the major late promoter (MLP) and are subsequently processed

to generate the later transcripts (L1 to L5). Other late transcripts include those from the late E2 promoter as well as the RNA polymerase III and transcribed RNAs.[33, 43, 46]]

1.5.3.2.2.2 Lytic cycle of adenovirus

Viral entry into the host cells occurs in two steps: attachment and internalization. Attachment to most cell types is mediated by binding of the fiber knob to host cell receptors, which appear to have some degree of subgroup specificity. Although the identities of the fiber receptors have not been definitively demonstrated, they are thought to include the $\alpha 2$ domain of the heavy chain of human major histocompatibility complex (MHC) class I molecules[47] and also a protein that also acts as a receptor for Coxsackie B viruses.[48] Internalization of the virus is promoted by binding of the penton base to α_v integrins on the cell surface, allowing the adenovirus to reach the endosome and avoid lysosomal degradation. Inside the endosome, a stepwise disassembly program takes place allowing the adenovirus to release its genome into the nucleus. During this process, the pH of the endosome decreases leading to the release of the fiber from the virion, and the dissociation of the penton bases. The resulting rupture of the endosome allows the DNA to escape from inside the degraded capsid and to enter the nucleus.[49] The uncoating process of the adenovirus starts immediately after internalization and ends about 40 minutes after infection with translocation of the adenovirus DNA into the nucleus. As early as 60 minutes after infection, the adenovirus begins to transcribe its genome in the host cell.[49] Adenoviral transcription, replication, and virus packaging take place within the nucleus of the infected cell.

The E1A transcription unit is the first adenoviral sequence to be expressed during viral infection.[43] Alternative splicing of the primary E1A transcript results in expression of two major E1A proteins that are involved in transcription regulation of the virus and are required for adenoviral replication under normal circumstances. In

addition, E1A stimulates the host cell to enter S phase and in parallel, apoptosis of the infected cell is blocked by inactivation of p53 through E1B-19 kDa protein.[43, 50, 51] E1B is also responsible for blocking host mRNA transport and stimulating viral mRNA transport through alternatively spliced transcripts.[43] Other functions identified with the E1 regions from subgroups A and B are the ability to induce tumors in animals, however, there are no known associations between adenoviruses and human malignancies.[52, 53]

The E2 region encodes proteins required for viral DNA replication such as a binding protein, DNA polymerase and the terminal protein precursor (pTP), which can serve as a primer for DNA synthesis.[43] Seven proteins have been identified originating from the E3 region, many of which are involved in evasion of the host immune system.[54] These proteins serve to bind MHC Class I molecules, preventing their presentation at the cell surface and subsequent activation of the immune system through cytotoxic T cells. Additionally, E3 proteins have been shown to decrease the immune response by inhibiting cytolysis and inflammation through the inhibition of tumor necrosis factor. Although E3 proteins are an integral part of the normal adenoviral life cycle, their presence does not appear to be required for virus growth in tissue culture, and adenoviral vectors have been engineered with deletions in this area.[46, 55, 56] The E4 region encodes at least six proteins, some of which function in DNA replication, late gene expression, and host protein shut off. The open reading frame 4 (ORF4) product indirectly down-regulates E1A expression, resulting in a drastic reduction in viral DNA accumulation in certain situations and has led to its genetic manipulation to control new viral vectors.[57]

Viral DNA replication begins within 8 hours postinfection. Replication initiates at either ITR with pTP serving as a primer for DNA synthesis. This process is then catalyzed by adenoviral polymerase. Standard strand displacement mechanism is

responsible for DNA replication along with adenoviral binding protein and polymerase as well as cellular factors.[46, 58] Late gene expression occurs after the onset of viral DNA replication. Due to the influence of E1 and E4 gene production, almost all of the protein being expressed in the cell at this time is viral. Transcripts from the MLP encode most of the virion structural proteins, including penton, hexon, and fiber (encoded by L2, L3, and L5, respectively). Virus production continues for about 2 days postinfection at which time lysis occurs, releasing up to 1000 infectious viral particles per cell.[46]

1.5.3.2.2.3 Engineered adenovirus vectors

Most adenoviral vectors have been engineered using derivatives of adenovirus serotypes 2 and 5. First generation vectors typically have deletions in the E1 region to render the vector replication-defective, thus limiting virus production and lysis of the host cell. Because the E1 region is required to propagate vector stocks, the deleted genes have been engineered into a stable human embryonic kidney cell-derived 293 line providing these genes in *trans*.[59] The E3 region, not required for *in vitro* virus propagation, has also been deleted in some first-generation adenoviral vectors to allow a greater cloning capacity. Because one of the normal functions of this region is evasion of the host immune response, some expression of the E3 region is desirable, and typically the entire region is not deleted. Given adenoviruses are capable of packaging a genome that is 5% larger than the wild-type genome, combining the E1/E3 deletions provides a cloning capacity around 8Kbp.

The biology of these first-generation vectors is such that significant early and late gene expression occurs after infection/transduction.[46, 60] This gene expression has been associated with induction of an immune response against the adenoviral vector.[61] This immune response can eliminate transgene expression due to recognition and clearance of the vector-infected cells, as well as prevent transgene

expression following repeat administration of the vector.[61-64] Additionally, adenoviral proteins that remain in viral vector preparations due to insufficient purification can also elicit an anti-adenoviral immune response.[65] Not necessarily specific to the vector but still limiting to duration of transgene expression, is an immune response against the transgene product specifically.[66] Attempts to rectify these problems have led to second-generation adenoviral vectors, "gutless" adenoviral vectors and immunomodulation.[46, 60]

Second-generation adenoviral vectors have focused on combating the immune response. Modifications have been made in the E1-deleted back bone of first-generation vectors, principally in the E2 and E4 regions in an attempt to further attenuate viral gene expression in transduced cells.[46, 60] For example, vectors deleted in both the E1 and E2A region have been constructed and were shown to persist for a longer period of time than their E1 deleted vector counterparts. [67] Although promising, further work improving the production of this type of vector has only recently begun. Further testing of the host responses to the virally transduced cells coupled with the evaluation of transgene expression times achieved in the absence of viral DNA replication (E2A) are also still ongoing. The E4 region, as previously stated, is in part responsible for subverting endogenous gene expression at different levels. E1/E4 deleted adenoviruses can be grown in culture and have been assessed *in vitro* and *in vivo*. Compared to E1 deleted vectors the E1/E4 deleted vectors provided a lower replication of the viral backbone thought to be a delay in replication events. Additionally, an almost complete shutoff of late gene and protein expression, as well as no apparent virus-induced cytotoxicity, provided optimism for this vector construct. However, despite these improvements over E1 deleted vectors further testing demonstrated transgene expression was not significantly improved.[68]

In another strategy to improve the performance of adenoviral vectors, removal of all transcription units from the viral backbone ("gutless") has been undertaken. Through the use of a helper virus, supplying most of the genes needed for packaging, gutless adenoviral vectors have been produced. Although technical difficulties exist in the production of this type of vector, further discussion of these difficulties are included in the following sections. As with E1/E4 deleted vectors, disadvantages to crippling the natural mechanisms of the adenoviral structure exist. Recent reports have shown beneficial results using this class of vector, specifically in increased transgene expression periods, and decreased immune and inflammatory responses.[69] Others have cited gutless adenoviral vectors to be associated with transient transgene expression, causing some to remain skeptical about their usefulness at this time.[60]

Immunomodulation has also been shown to be an effective means of extending the transgene expression time and decreasing the immune and inflammatory reactions to adenoviral transduced cells. The use of oral adenoviral antigens, cyclophosphamide, cyclosporine A and specific anti-T cell molecules to decrease the immunogenic response have led to extended transgene expression for up to 300 days.[30, 60, 70] Additionally, through the use of immunosuppression, repeat administration of adenoviral vectors has been possible without either evoking deleterious immune responses or shortened transgene expression times. Temporarily crippling the host's natural defense system in order to hide a vector from immune modulated attack is obviously not without potential complications. However, it may provide a short-term solution for the use of adenoviral vectors in gene transfer protocols allowing time for more sophisticated vectors to be engineered.

Another consideration and limitation of adenoviral vectors revolves around the strategy used for the construction of the vector. The optimal construction of a recombinant vector would provide for easy insertion of a transgene, produce a high titer

stock of the recombinant vector, be free of non-essential viral components and be amenable to large-scale production. The production of many first-generation adenoviral vectors utilize homologous recombination of a shuttle vector rescued following co-transfection of either a circular plasmid containing the adenoviral genome or purified adenoviral DNA that has been cleaved by restriction enzymes to reduce the recovery of wild-type virus that has regained its ability to replicate (replication-competent adenovirus (RCA).[46] RCA contamination can be deleterious for several reasons: (1) RCA can direct high levels of viral gene expression leading to immune and inflammatory reactions in addition to cytotoxic events. (2) RCA can also act as a helper-virus and increase the effective dose of the therapeutic vector as well as contribute to the mobilization of the therapeutic vector. Methods to overcome some construction limitations of first-generation adenoviral vectors have been made using helper-dependent adenoviral vector systems. This system retains many of the advantages of first-generation adenoviral vectors, mainly high transduction efficiency of both replicating and non-replicating cells, but also has the added advantages of increased safety (by decreasing RCA production). This technology is also used in the construction of "gutless" systems and provides advantages in the increased cloning capacity of the vector and potential for reduced immune responses through the elimination viral gene transcription. Because of the uncertain *in vivo* performance of gutless vectors at the onset of these studies, the authors chose to utilize a helper-dependent adenovirus vector system that utilized Cre-mediated excision of the helper virus to create an E1/E3 deleted adenoviral vector.

This Cre system is based on the P1 bacteriophage Cre-*loxP* system., The Cre gene encodes for a recombinase that facilitates the recombination between two parallel *lox* sites (present in one DNA strand) resulting in excision of the intervening sequence, producing two recombination products each containing one *lox* site. In this case the helper virus is designed to have two *loxP* sites on either side of the packaging signal

(required for efficient packaging of the viral genome) (Figure 3). When this helper virus is used in CRE8 cells (a 293 cell line derivative making a high concentration of Cre recombinase) recombination will delete the intervening packaging sequence producing an unpackageable viral genome. When generating recombinant virus, the ψ5 virus is used as a donor for the viral backbone. In the context of the CRE8 cells there is negative selective pressure against the propagation of ψ5 viral backbone. Furthermore, the Cre recombinase catalyzes recombination between ψ5 and a shuttle vector with a single *loxP* site and a transgene, providing an efficient means to construct recombinants. (Figure 3).[71]

1.5.4 Possible anti-arthritic agents

At the onset of these studies, a vast majority of possible anti-arthritic agents existed and many more have since been described.[72] As outlined in previous sections, the goal of these studies was to evaluate the utility of gene transfer as a methodology to treat joint disease. Although the specific gene sequence being expressed using this methodology is important, the flexibility of the method to allow the expression of other described and novel gene sequences was equally important to this work. The specific gene sequence chosen was based on its previous characterization in the horse and historical information regarding its anti-arthritic potential in other species. The following section will briefly cover some of the known pathophysiology of joint disease and provide some other potential techniques for therapeutic intervention. This will be followed by a more in-depth discussion of the agonist and antagonist potential of interleukin-1 molecules. This discussion knowingly excludes a vast majority of other potential anti-arthritic agents that are beyond the scope of this manuscript.

1.5.4.1 Etiopathogenesis of Osteoarthritis

Joint disease has been defined as a complex of interactive degradation and repair processes in cartilage, bone and synovium, with inflammation playing a pivotal role in the degradation processes.[73] The specific contribution from each of these joint tissues may differ depending on specific disease processes within the joint. However, some level of involvement from all is likely present in most cases. Although not the only mediators of joint disease, the interleukins and tumor necrosis factors are recognized as centrally involved in the genesis and maintenance of inflammatory responses, and are thought to be key molecules in mediation of most joint disease.[8, 74, 75] The activity of interleukin-1 (IL-1) overlaps largely with that of TNF and certain other cytokines. Because these studies focus on blocking IL-1 activity through the use of an interleukin-1 receptor antagonist, the following discussion will focus on IL-1.

1.5.4.2 Role of IL-1 system

IL-1 is the term for two polypeptide mediators (IL-1α IL-1β) that are among the most potent and multifunctional cell activators described in immunology and cell biology. The spectrum of action of IL-1 encompasses most cell types, and occupancy of only a few receptors has been described to be sufficient to elicit cellular responses.[76-78] The production and action of IL-1 is regulated by multiple control pathways, some of which are unique to this cytokine. The IL-1 "system" consists of the two agonists IL-1α and IL-1β, a specific activation system (interleukin-1 converting enzyme, ICE), a receptor antagonist (IL-1Ra) produced in different isoforms,[79] and two high-affinity surface binding molecules (type I and type II receptors, Figure 4).[80]

1.5.4.2.1 IL-1α & IL-1β

Human IL-1α and IL-1β share 45% homology at the nucleotide level, while the similar equine sequence share 26% homology. Sequence homology of each molecule between species is relatively high, ranging from 60% – 80%.[81, 82] A variety of stimuli have been shown to activate and act synergistically in the transcription of IL-1 genes. Among these are endotoxins from Gram-negative and exotoxins from Gram-positive bacteria, UV light, T cells, and complement components to name just a few.[81] Among cytokines, IL-1 itself, TNF, and IL-2 have all been shown to up-regulate IL-1 gene transcription.[83-86] IL-1 gene transcription is also under negative control, influenced by other cytokines such as IL-4, IL-6, IL-10, and IL-13, as well as glucocorticoids. Post-transcriptional regulation is a further level of control of IL-1 expression.[81] Inherently, in some circumstances mRNA of IL-1α compared to IL-1β is less stable.[87] Other cytokines, such as IL-4 have been shown to destabilize IL-1 mRNA, thus acting to negatively regulate IL-1 expression.[88] When translated, the IL-1β precursor is not biologically active and relies on ICE mediated cleavage of pro-IL-1β to mature IL-1β for activity. Although it was once thought that no pro-form of IL-1α existed[89], recent evidence indicated that this molecule is translated without a signal peptide as a 31-kDa precursor (pIL-1α). pIL-1α undergoes proteolytic activation to release the mature carboxyl terminus 17-kDa protein (mIL-1α). Both the pIL-1α and mIL-1α proteins are biologically active. Interestingly, only pIL-1α contains a nuclear localization sequence, which once having cleaved the N-terminal pro-piece can act as a transforming oncoprotein in certain cell types.[90] Furthermore the pIL-1α has shown the ability to modulate cell migration similar to IL-1Ra further suggesting an intracellular or "intracrine" mode of action.[91] This has led some researchers to postulate intracellular signaling triggered by IL-1 may involve biochemical pathways that by pass the prerequisite of IL-1 binding to its cell surface receptor.[92]

There have been many studies demonstrating the varied effects of human recombinant IL-1 on articular cartilage in a number of different species. Relatively little information exists specifically describing IL-1 effects on equine cartilage and even less information exists on the effects of equine IL-1 agonist proteins on equine articular cartilage. Noted effects of IL-1 include suppression of proteoglycan (PG) biosynthesis, enhancement of PG degradation, and increased prostaglandin E_2 (PGE_2) release, all in numerous species.[93-97] Studies have also shown the ability of IL-1 to up-regulate the production of mediators of cartilage degradation in cultured cartilage and synoviocytes *in vitro*, specifically matrix metalloproteinase (MMP) activity for MMP-1, MMP-3 and 13 as well as PGE_2, and IL-6.[98-103] The role of IL-1 in experimentally created and naturally occurring joint disease is also established in many species, including the horse.[104-108]

1.5.4.2.2 IL-1 receptors

The type I IL-1 receptor is a molecule which mediates the known biological responses to IL-1.[109] The cytoplasmic domain of the receptor is necessary for the signaling function of the receptor, and utilizes both the transcription factor NFκB and MAP (mitogen-activated protein) kinase pathways.[110, 111] Recombinant IL-1 soluble type I receptor has been shown to be an effective antagonist of IL-1 action, both *in vitro* and *in vivo*.[112, 113] The IL-1 type II receptor appears not to deliver biological signals, and therefore serves to trap IL-1 and inhibit its action by preventing its interaction with the type I receptor. The extracellular ligand binding region of the molecule is similar to that of the type I receptor, being comprised of three immunoglobulin-like domains, although this region only shares a 28% homology in amino acids. The transmembrane and cytoplasmic portions of the type I and type II receptors share no similarity. The distribution of the type II receptors is more restricted than type I and appears to be more

actively regulated.[114] For example, IL-4 and glucocorticoids lead to strong upregulation of type II receptors.[115, 116] Type II receptors not only block IL-1's biological activity as a surface receptor, but the molecule can also be solubilized subsequent to proteolytic cleavage or as an alternatively processed mRNA molecule and serve to block IL-1 activity.[117, 118] Interestingly the type II receptor gene is encoded in the genome of certain pox viruses, and blockade of cellular IL-1 by the virally-encoded receptor affects viral pathogenicity.[119] As expected, the IL-1 receptors have different affinities for the IL-1 ligands. The type I receptor has the highest affinity for IL-1α and IL-1Ra (K_D about 10^{-10}M) while having a lower affinity for IL-1β (K_D about 10^{-9}M). The type II receptor has the greatest affinity for IL-1β (K_D about 10^{-9}M) while having a similar lower affinity for IL-1α and IL-1Ra. (K_D about 10^{-8}M).[120] The pattern of ligand/receptor affinities may suggest that IL-1Ra and the type II receptor function as inhibitors of IL-1α and IL-1β respectively.

1.5.4.2.3 IL-1 receptor antagonist

Since the early 1980s proteins from human patients with specific anti-IL-1 action have been described.[121, 122] The 23 – 25 kd IL-1Ra protein was purified from the urine of patients with myelomonocytic leukemia and the cDNA from this molecule was cloned soon after.[123-125] The agonist effects are partly regulated by the interaction between IL-1Ra and the IL-1 receptors. To date, four different forms of IL-1Ra have been described, all transcribed from the same gene but generated by alternative splicing or translation start sites.[92] The secreted form of IL-1Ra (IL-1sRA) was the first form described, and it binds to IL-1 receptors blocking the binding of the other IL-1molecules.[126] No other function has been assigned to IL-1sRa.[81] The other forms of IL-1Ra exist as intracellular forms raising questions concerning possible intracellular signaling routes that involve mature or unprocessed IL-1. The intracellular forms are

designated as type I and type II IL-1iRa, while the fourth form has a designation of IL-1iRa$_{exon3}$.[92] The function of the intracellular IL-1Ra forms remain poorly characterized, but suggests that a more complex system for IL-1 regulation exists. IL-1α, IL-1β, IL-4, IL-10, IL-13 all result in the induction of IL-1Ra and different stimuli and levels of cellular maturation also have varied effects on IL-1Ra induction.[81]

1.5.4.2.3.1 Therapeutic potential of IL-1Ra

The beneficial clinical implications of blocking the activity of IL-1 are many and extend to: vascultis, disseminated intravascular coagulation, osteoporosis, osteoarthritis, neurodegenerative disorders such as Alzheimer's disease, diabetes, lupus nephritis, immune complex glomerulonephritis and autoimmune diseases in general.[77] Inhibition of IL-1 by IL-1Ra has been shown to be protective in various animal models including endotoxin-induced hemodynamic shock, arthritis, inflammatory bowel disease, spontaneous diabetes in rats, graft-versus-host disease in mice, heart allograft rejection, and experimental autoimmune encephalomyelitis. Results from human clinical trials using IL-1Ra therapeutically are now available and although a phase II randomized placebo-controlled clinical trial with IL-1Ra treated patients suffering from sepsis showed a dose-dependent improvement in 28-day survival, a subsequent phase III study failed to substantiate the phase II data.[127, 128] Although secondary analysis of a subset of the data lead to rekindled optimism that IL-1Ra may have been beneficial in patients with more serious disease and organ failure, increasing their survival time, a later study refuted any measurable beneficial effects using a similar patient population.[129] The therapeutic effects of IL-1Ra in joint related diseases has fortunately been more promising.

IL-1Ra has been studied in synovial fluid from patients with a variety of joint related problems. Levels were elevated in synovial fluid from patients with rheumatoid

arthritis (RA), in 1/3 of samples from patients with other infectious or inflammatory arthropathies, and in none of the non-inflammatory control samples.[130] This study also showed a strong correlation between IL-1Ra levels and polymorphonuclear neutrophil (PMN) concentrations. Elevation of IL-1Ra in inflammatory synovial fluid samples may appear paradoxical, as IL-1Ra is considered to be an inhibitor of IL-1 induced inflammation; however, the ratio of IL-1 to IL-1Ra appears to be important in the biological response of the tissues. Studies have suggested that less than 5% of the IL-1 type I receptors need to be occupied by IL-1 to induce a biological response and that 10 – 100-fold excess amounts of IL-1Ra are required to inhibit 50% of the IL-1 response in cells that express the IL-1 receptor.[78, 79] Further support of this can be seen in patients with acute Lyme arthritis. The duration of joint inflammation was least in those patients with the highest joint fluid levels of IL-1Ra and greatest in those with low levels of IL-1Ra.[131] However, results of a dose titration study performed in humans using subcutaneous injection of IL-1Ra in RA patients, combined with results of preliminary gene transfer experiments, suggests a large molar excess of IL-1Ra may not be required to inhibit arthritic progression.[19, 23, 132] Specifically, conclusions reached through work performed by Campion and colleagues,[19] states frequency of IL-1Ra administration may be more important than dosage, suggesting a key role for sustained protein expression through gene transfer. Furthermore, gene transfer studies have demonstrated improvement in parameters of joint degeneration when peak IL-1Ra expression of 3.88 ng/mg total protein within the synovial fluid was measured in response to stimulation by 25 ng of IL-1 protein. [132]. Similar work, using a retroviral vector, demonstrated the ability of approximately 5 ng of IL-1Ra to inhibit the response to 1 and 5 ng of IA injected IL-1β.[24] When an antigen-induced arthritis model in rabbits was utilized as an *in vivo* RA model, IL-1Ra expression through gene transfer at 15 ng/joint demonstrated the ability to inhibit pathologic parameters of joint disease. In the

same study, independent quantification of IL-1β showed peak levels to be less than 150 pg/joint, at most a 100-fold molar difference.[19] These results provide evidence that a large molar excess of IL-1Ra versus IL-1 may not be required in the treatment of joint disease through gene transfer methods.

The administration of IL-1Ra protein in experimental arthritis has also dramatically impacted the course and outcome of *in vitro* and *in vivo* models of arthritis. Administration of IL-1Ra protein has shown the ability to inhibit prostaglandin production by chondrocytes and synovial cells, and collagenase production by IL-1 activated synovial cells.[133] The effects in cartilage of both IL-1 agonists were suppressed in a concentration-dependent manner by human recombinant IL-1Ra.[134] IL-1Ra has also been shown to suppress IL-1 activated MMP and PGE production by chondrocytes.[134] Intravenous administration of IL-1Ra has also shown protective effects in joint tissues after intra-articular IL-1 injection,[135] as well as intra-peritoneal IL-1Ra administration blocking inhibition of proteoglycan synthesis and monocyte infiltration into synovial tissues in murine-immune complex arthritis.[136] The intra-articular administration of human recombinant IL-1Ra in a Pond-Nuki model of OA in dogs demonstrated a dose dependant reduction in the incidence of femoral condyle osteophytes, size and grade of tibial plateau cartilage lesions, significantly less severe cartilage histology and reduction in MMP-1 expression.[137]

The efficacy of IL-1Ra protein administration for the treatment of human RA was recently evaluated in a 24-week placebo-controlled double-blind randomized study.[138] The study had stringent entry criteria, such as at least 10 swollen joints and severe or very severe disease activity. Following 24 weeks of treatment with placebo or 3 different concentrations of subcutaneously administered IL-1Ra (ranging 30 –150 mg/day) statistically significant improvements in clinical parameters compared to placebo were observed in patients receiving the highest tested IL-1Ra concentration. These clinical

parameters included number of swollen joints, investigator assessment of disease activity, patient assessment of disease activity, pain score, and duration of morning stiffness. Additionally of particular interest, was a significant reduction in radiographic progression of joint disease (identified pre-treatment) from IL-1Ra compared to placebo treated patients at 24 weeks as determined by serial Larsen scores.[139] Also the radiographic progression measured by number of new joint erosions was demonstrated to be significantly less in the IL-1Ra treated patients as compared to the placebo group. Unfavorable effects noted in human trials involving daily injections of IL-1Ra protein have been transient injection site reactions leading to around a 12% withdrawal rate. To obviate daily administration and potentially maintain a more consistent level of IL-1Ra therapy, researchers have explored the expression of the IL-1Ra gene as a method of IL-1Ra administration.

1.5.5 Gene transfer of IL-1Ra

One of the first reports demonstrating beneficial effects of gene transfer using the IL-1Ra gene sequence was published in 1993. In this study, a retroviral vector was used through *ex vivo* gene transfer techniques to produce a decreased synovial fluid leukocyte influx into treated joints injected with IL-1β, as compared to placebo treated joints.[24] Using a similar model, continued work by this group demonstrated the ability of IL-1Ra to also inhibit synovial membrane thickening, and hypercellularity and articular cartilage proteoglycan loss.[25] Using a different model (rabbit antigen-induced arthritis) the same group confirmed decreased synovial fluid leukocyte infiltration, however, other measured inflammatory parameters, such as joint circumference, were not improved. Significant inhibition of matrix synthesis and decreased matrix catabolism were noted in the articular cartilage of treated versus placebo treated joints based on proteoglycan synthesis and degradation.[19] Others still working with retroviral vectors, but using a

bacterial cell wall-induced arthritis in rats, found contradictory results, with improvements in joint swelling without attenuation of cartilage erosion.[140] Work in the dog, a species with naturally occurring joint disease, demonstrated a marked reduction in macroscopic and histologic lesions in the tibial plateaus and femoral condyles of cruciate deficient subjects expressing the human IL-1Ra gene sequence as compared to control joints.[141] In 1996, Evans and colleagues became the first to commence a clinical trial with a retroviral vector assessing the safety, feasibility, and efficacy of IL-1Ra within human joints affected with rheumatoid arthritis.[28] Adenoviral based vectors have also been tested and have shown the human IL-1Ra gene sequence to protect against IL-1 induced proteoglycan degradation in articular cartilage, through *in vivo* gene transfer in rabbits.[132] Other gene sequences such as TNF soluble receptors, IL-4 and IL-10 have been used to combat joint deterioration. However, an inclusive review of other therapeutic gene sequences is beyond the scope of this manuscript and can be found in other references.[72]

1.5.6 Conclusion

The ability to transfer anti-arthritic gene sequences directly to the joint space is available today. When hurdles currently being addressed in the field of gene therapy vector engineering are overcome, such as site/cell specific transductions[142, 143] and disease-associated transgene regulation[144], the optimal gene therapy vector system will hopefully be realized for the treatment of joint disease. This coupled with further research into the pathogenesis of joint disease, providing insights into which gene sequence(s) may be lacking or beneficial when over-expressed, will hopefully lead to either a cure or dramatic reduction of the devastating progression of joint disease. The studies presented here are an attempt to bring the state of the art joint disease treatment

to the equine species, in hopes that when the previously stated technologies are realized, the horse will be ready.

CHAPTER 2

2 Construction, testing and *in vivo* dose titration of an adenoviral vector expressing the equine IL-1Ra gene sequence

2.1 Introduction

Joint disease is a severe mobility and career modifying disease in humans and horses. The etiopathogenesis of joint disease is not clearly understood. However, many mediators that lead to degeneration within the joint environment have been identified. Cytokines, especially interleukin-1 and tumor necrosis factor, have been defined as initiating mediators of a degenerative cascade that propagates joint disease.[145, 146] Through the use of anti-cytokine proteins, it has been demonstrated that blocking these molecules can significantly alter the course of joint disease. Many of these studies, however, have been performed on experimentally created joint disease in laboratory species. The effects on species with naturally occurring disease or the long-term outcomes of treatment using anti-cytokine therapies have not been adequately studied.[72, 141, 147, 148] While the studies presented here will not evaluate the efficacy of treatment for joint disease they will provide proof of principle that gene therapy as a treatment modality using an anti-cytokine gene sequence is feasible in a species with naturally occurring joint disease.

As previously stated, many anti-arthritic agents have been identified. Interleukin-1 receptor antagonist (IL-1Ra) was chosen as the disease modifying gene sequence in these experiments for three reasons: (1) A significant amount of work has been done in various species demonstrating IL-1Ra's ability to modify joint disease. (2) Preliminary work has been completed using both the protein given by direct intra-articular injection, as well as expression of the IL-1Ra gene sequence through various gene transfer protocols. (3) The horse is an athletic species with naturally occurring joint disease and the equine gene sequence for IL-1Ra[149] was known at the onset of these studies.

Over the last decade, gene transfer has become an accepted and even preferred method of continuous protein expression targeting certain disease conditions, although the optimal vector for use in gene transfer is still undetermined.[30, 31, 34, 150] Early in the genesis of gene transfer protocols, the use of retroviral vectors predominated for various practical and safety reasons. Some of these reasons included the ability to harvest cells, transduce them *in vitro* and, after safety testing, introduce the altered cells into the patient. The benefits of stable integration of the vector's genome, once pronounced as a major reason to use *ex vivo* gene transfer, has not been realized as a clear advantage for *ex vivo* methods.[34] Recent trends indicate the growing use of vectors that can be delivered using *in vivo* gene transfer methods.[29, 32, 40] *In vivo* administration obviates the harvesting, *in vitro* transduction of cells, and re-implantation to the patient that is required using *ex vivo* methodology. The ability to perform extensive safety testing is partially sacrificed using *in vivo* gene transfer, and although significant safety issues have been proposed they have yet to be identified. However, some limitations must be overcome prior to the wide spread use of gene therapy in clinical disease states. Specifically as it relates to the treatment of joint disease, the lack of long term expression and regulation of transgenes must be overcome. However, research addressing these limitations continues and shows great promise for the future.

The use of an adenoviral vector in the studies experiments described herein was based on the factors outlined in the previous discussion, extensive characterization of the vector, its use in laboratory animals, and its clinical ease of application.

This manuscript is a compilation of experiments demonstrating the ability to deliver interleukin-1 receptor antagonists using *in vivo* gene transfer in a species with naturally occurring joint disease. The goal of this research was to test a modality believed to have a clinically relevant application in the treatment of joint disease, prior to evaluation using an experimentally created model of equine joint disease. The specific aims of the *in vitro* studies were; (1) To establish the transduction efficiency of equine synoviocytes using characterized vectors carrying marker genes. (2) To establish the detectable level and biologic activity of equine IL-1Ra produced after in vitro transduction of equine synoviocytes using an adenoviral vector carrying the equine IL-1Ra gene (Ad-EqIL-1Ra). (3) To establish that equine IL-1Ra produced after in vitro transduction of equine synoviocytes was an active protein. The specific aim of the *in vivo* studies was to establish the *in vivo* concentration and duration of expression of equine IL-1Ra for five different concentrations of the Ad-EqIL-1Ra vector.

2.2 Materials and Methods

2.2.1 In vitro cell culture

Synovium was aseptically harvested from grossly normal metacarpophalangeal joints of 5 horses ranging in age from 10 – 15 years. Prior to processing, synovium was stored in phosphate buffered saline (PBS) at 4C unless otherwise noted. Synovium was further divided into $3mm^2$ pieces and placed in Ham's F12 media with 0.2% collagenase and cultured for 4 hours in a 37 C, 5% CO_2 environment with gentle agitation. Each liter of standard Ham's F12 was supplemented with 25ml HEPES, 200ml fetal calf serum,

50mg ascorbic acid, 300mg L-glutamine, 30mg α-ketoglutaric acid, and 20,000IU penicillin and streptomycin. After collagenase digestion, the cell mixtures were strained using 4-ply cheesecloth and cell numbers were quantified. Cells (5×10^5 cells/flask) were cultured in $25cm^2$-culture flasks containing 4ml of supplemented media in a 37 C and 5% CO_2 environment. Media was changed at 7-day intervals unless otherwise noted.

2.2.2 In vitro transductions

All cells were grown to ≈ 60-70% confluency before transduction experiments were started. Prior to transductions, flasks were rinsed twice with sterile phosphate buffered saline (PBS) and 2mls of serum–free supplemented media were added to each flask. The appropriate volume of viral preparation was then added to each flask, followed by gentle rocking in a 37 C, 5% CO_2 environment for 4 hours. Cells were then rinsed twice with PBS and serum containing supplemented media added to the flasks. The cells were maintained in a 37 C, 5% CO_2 environment unless otherwise noted.

2.2.3 Vectors

A helper-dependant adenovirus vector system, utilizing Cre-mediated excision of ψ5 helper virus to create an E1/E3 deleted adenoviral vector containing the equine gene sequence for secreted IL-1Ra, was used in the creation of Ad-EqIL-1Ra.[71] Using routine molecular biology techniques[151] the gene sequence (Accession, U92482) was engineered to contain BamHI restriction sites on either side of the coding sequence (nt 14 – 548) using upstream (5'-GGTTGT**GGATCC**AGGATGGAAATCCGCAGG) and downstream (5'-GTCTCT**GGATCC**ATCGACATGCTGGGAATAGG) PCR primers containing a BamHI site shown in bold. The PCR products were digested with BamHI, purified from a 1.5% agarose gel and ligated into the pAdlox adenoviral shuttle

plasmid.[71] After restriction digestion of the pAdlox plasmid with SfiI, recombinant virus was generated by co-transfection of the digested DNA and the ψ5 adenoviral helper virus into CRE8 cells. Plaques were isolated, expanded and characterized for insertion of the eq-IL-1Ra cDNA and its expression. To generate stocks of virus, confluent flasks of CRE8 cells were infected with the Ad-EqIL-1Ra virus. After detection of significant cytopathic effects, the cells were harvested, pelleted, re-suspended in 5ml of saline and stored at –80C. To purify the virus the cell pellet was lysed by three rounds of freeze-thaw. The cell debris was pelleted by centrifugation, and the cleared lysate was collected. Virus was banded three times over successive cesium chloride step gradients. After dialysis the virus was collected, aliquoted and stored at –80C until use.

Construction of the adenoviral vectors encoding human IL-1Ra (Ad-HuIL-1Ra) and E. coli β-galactosidase (adLacZ) transgenes have been previously described.[152]

2.2.4 Lac-Z, IL-1Ra or PGE_2 detection

Three days post adLacZ transduction cultured cells were stained with X-gal (5-bromo-4-chloroindolyl-β-D-galactose).[152] X-gal turns a blue color in the presence of β-galactosidase and therefore can be used as a marker of transgene production. The number of cells stained blue were counted and expressed as a percentage of the total cells counted (n=500). Concentrations of IL-1Ra and PGE_2 were estimated from collected media stored at –80 C. Media aliquots were used for determination of IL-1Ra utilizing a commercially available kit and manufacture's recommendation.[i] Media aliquots were used for determination of PGE_2 utilizing a commercially available kit and manufacturer's recommendation.[ii]

2.2.5 Conventional synovial fluid analysis

Synovial fluid color, clarity and mucin content were evaluated subjectively, and total protein and inflammatory cell (WBC) concentrations were determined by use of routine clinicopathologic methods.[iii] Color of synovial fluid was graded as yellow, colorless, straw, orange or red and numeric values of 1 – 5, respectively, were assigned. Clarity was graded as clear or cloudy and numeric values 1 or 2, respectively, were assigned. Mucin content was graded as good, fair or poor and numeric values of 1 – 3, respectively, were assigned.

2.2.6 Joint effusion and lameness examinations

Evaluation of joint effusion and lameness were conducted as previously described.[153, 154] Briefly, prior to synovial fluid arthrocentesis, joints were subjectively graded as having normal, slight, mild, moderate, or severe synovial effusion. This grade was converted to a numeric value (0 – 4), with 0 being normal and 4 severe, and used in the statistical analysis. Similarly, lameness examinations were performed on the horses prior to arthrocentesis. Each limb was assigned a grade of lameness according to a published scale.[155] As well as, the carpal and metacarpophalangeal joints were flexed in an attempt to exacerbate any pain emanating from the region. The response to flexion was graded similarly to joint effusion.

2.2.7 Experimental design

2.2.7.1 In vitro

Two flasks of synoviocytes were transduced with the adLacZ vector at a multiplicity of infection (MOI) of 0, 1, 10 and 100 to determine transduction frequencies. Two flasks of synoviocytes were infected with Ad-EqIL-1Ra at 0, 1, 10, and 100 MOI to

determine the concentration of IL-1Ra produced at the respective vector doses. After transduction, the cells were cultured for an additional 48 hours after which time the media were removed and stored (-80 C) and the cell numbers in each flask were estimated using a hemocytometer. The concentrations of IL-1Ra in the media samples were normalized to 1 x 10^6 cells and a 48-hour culture period. To determine if equine synoviocytes transduced with the Ad-EqIL-1Ra vector produced an active protein, a bioassay was performed. Synoviocytes were either non-transduced or transduced (Ad-EqIL-1Ra) at 10 MOI, cultured for 2 days, and then 10ng of human recombinant IL-1α[iv] was added to the media of some flasks followed by an additional 2 days of culture. The media were collected and stored (-80 C) for IL-1Ra and PGE_2 determination, and the cell numbers in each flask were eliminated.

2.2.7.2 In vivo

Six horses were utilized for determining concentration and duration of IL-1Ra production following *in vivo* transduction of equine midcarpal and metacarpophalangeal joints at various doses of Ad-EqIL-1Ra (ranging from 1 x 10^9 – 5 x 10^{11} particles/joint). Each limb had one virally transduced joint while the same joint on the other limb served as the placebo treated control (receiving a similar volume of diluent). Therefore, each horse had 2 transduced joints on opposite limbs. The Ad-EqIL-1Ra stock solution (1 x 10^{12} particle/ml) was diluted in Gey's balanced salt solution[iv] for a final volume of 1000µl. The diluent was chosen as the placebo to test the antigenicity of both the vector and proteins produced in association with its presence. Each viral dose (1 x 10^9, 1 x 10^{10}, 1 x 10^{11}, 2 x 10^{11}, & 5 x 10^{11} particles/joint) was tested in two horses. At the time of transduction the transduced and placebo joints were aseptically prepared and arthrocentesis performed using a syringe and 20ga x 1.5" needle. Approximately 4ml of synovial fluid was collected for routine synovial fluid analysis (total protein, WBC counts,

differential cell count, color estimation, and quality of mucin clot) and IL-1Ra quantification. The synovial fluid samples were split into tubes containing EDTA (routine synovial fluid analysis) or sodium citrate (IL-1Ra determination). During the same procedure either the vector or placebo treatment was administered directly into the joint cavity. Arthrocenteses were repeated on days 3, 7 and at 7 day intervals thereafter until day 35 post transduction or until measured IL-1Ra protein was similar to control levels. Previous work by others documented some difference in synovial fluid volume between the midcarpal and metacarpophalangeal joints (14.9± 0.6ml and 12.5 ± 1.0ml, respectively) of the horse.[156] Due to this small volume difference, the synovial volumes of both joints were considered similar for the purposes of this study.

2.2.8 Statistical analysis

Results of the in vitro experiments were analyzed using a one-way ANOVA. Dependant variables used in the analysis were transduction efficiencies and protein concentrations (IL-1Ra & PGE_2). The vector concentration was used as the independent variable. In all analyses, a p-value < 0.05 was considered significant, and residual plots made to test for fulfillment of model assumptions. If model assumptions were not met data transformation was performed and reported. Synovial fluid samples were analyzed using mixed-model ANOVA.[157] Dependent variables were synovial fluid IL-1Ra, total protein and WBC concentrations, color, clarity, and mucin clot scores, percentage of the WBC count contributed by neutrophils, monocyte and lymphocytes, degree of joint effusion, lameness and response to joint flexion. Independent variables were joint location (intercarpal or metacarpophalangeal joints), treatment (administration of Ad-EqIL-1Ra or placebo), day of sample collection, and all interactions between main effect variables. When individual comparisons were made, a Least Squares Mean procedure

was utilized, a P-value ≤ 0.05 was considered significant.[158] All subjective evaluations were completed by evaluators blind to treatment assignments.

2.3 Results

2.3.1 In vitro

Transduction efficiencies in equine synoviocytes were correlated to MOI of the adLacZ vector (Table 1). Equine synoviocytes transduced with Ad-HuIL-1Ra demonstrated a significant increasing dose response in measured IL-1Ra released into the media (data not shown). Likewise, a similar increasing dose response in the concentration of IL-1Ra measured in the media was seen after Ad-EqIL-1Ra transduction of equine synoviocytes at various MOIs. (Figure 5).

Cells exposed to human IL-1α (10ηg/ml of media) for 48 hours had significantly higher PGE_2 levels in their media if they were non-transduced as compared to transduced (Ad-EqIL-1Ra) cells (Figure 5). Transduced cells exposed to IL-1α had similar PGE_2 levels to cells not exposed to IL-1α, either transduced or non-transduced (Figure 6). Similar results were observed at 100 MOI concentrations of the Ad-EqIL-1Ra vector, however, at concentrations lower than 10 MOI protection against IL-1α stimulated PGE_2 production was not seen (data not shown).

2.3.2 In vivo

When the Ad-EqIL-1Ra vector was administered at a dose of 0.1×10^{10} particles/joint, no difference in synovial fluid IL-1Ra concentrations could be observed when placebo and transduced joints were compared. At doses of $1.0 \times 10^{10} - 50 \times 10^{10}$ particles/joint, a significantly higher concentration of IL-1Ra was detected within synovial fluid of joints, transduced with the Ad-EqIL-1Ra vector compared to placebo treated

joints (Figure 7). Duration of IL-1Ra detection within the synovial fluid of joints transduced at both 10 and 20 x 10^{10} particles/joint was significantly longer than for joints transduced with 50 x 10^{10} particles/joint (Figure 7). An interesting finding in joints transduced at 50 x 10^{10} particles/joint was a low but significant increase in measured IL-1Ra levels after day 21 post-transduction (Figure 8). This increase was seen in both the transduced and placebo treated joints and was statistically significant at day 35 in both joints as compared to the placebo treated joints from days 0-14. Similar results were not seen at other tested doses.

Routine clinical pathologic analysis of synovial fluids indicated that a significant decrease in the quality of mucin clot was demonstrated after repeat arthrocenteses. The mean score ± standard error on day 0 for all joints (placebo and transduced) was 1.4 ± 0.2 compared to 2.2 ± 0.3 on day 35. The change in mucin clot score was independent of treatment group (placebo or transduced), meaning the change was a result of the arthrocentesis procedure. At the highest Ad-EqIL-1Ra concentration (50 x 10^{10} particles/joint) a significantly greater synovial fluid WBC concentration was observed on days 3 and 7 as compared to synovial fluid from joints with lower Ad-EqIL-1Ra concentrations at similar time periods (Figure 9). A change in the synovial fluid cell population was observed at different concentrations of the Ad-EqIL-1Ra vector. A higher percentage of neutrophils (Figure 10) and a lower percentage of mononuclear cells (data not shown) were observed in the synovial fluid of joints transduced with 0.1 and 1 x10^{10} particles/joint. The percentages of neutrophils and mononuclear cells were independent of the sampling time period. Also a trend (P-value >0.05) for a lower percentage of synovial fluid lymphocytes was observed in synovial fluid samples collected from all joints at day 35 post transduction, compared to all other days except on day 7 where a

similar trend existed (Figure 11). The changes in synovial fluid lymphocyte concentrations were independent of administration of Ad-EqIL-1Ra vector.

2.4 Discussion

2.4.1 In vitro

Transduction efficiencies in equine compared to rabbit synoviocytes using the AdLacZ vector were lower than expected by ≈ 50 fold. In rabbits the AdLacZ vector has shown 100, 85, and 7% transduction frequencies at MOIs of 10, 1.0, and 0.1, respectively without cytotoxic effects being noted.[152] Although the MOIs were higher to achieve similar transduction efficiencies in equine synoviocytes, this higher level was not associated with observed cytotoxic effects. A difference in binding, internalization, transport, transgene expression or viral stock preparation could account for the species variations. Although no deleterious effects were noted *in vitro* with the higher viral particle load needed to obtain similar transduction efficiency (≈ 100%) in equine and rabbit synoviocytes, different results may occur *in vivo* and will need to be tested in future studies.

A dose response was seen in synoviocyte IL-1Ra production with increasing MOIs for the Ad-EqIL-1Ra vector (Figure 5). IL-1Ra concentrations measured from media of transduced cells were similar to those reported from other studies.[28, 159] Based on further work by these authors using similar vector constructs and *in vivo* models, the level of IL-1Ra observed in this study is expected to result in protective effects on diseased joint tissues.

The addition of human recombinant IL-1α to the culture medium of equine synoviocytes significantly increased PGE_2 concentrations in the media within 48 hours. The transduction of synoviocytes using Ad-EqIL-1Ra prior to the addition of the IL-1α

halted an increase in synoviocyte PGE_2 production after IL-1α exposure. The results of this simple bioassay suggested the transgene expression and secretion of a biologically active equine IL-1Ra protein. A similar bioassay was used to evaluate transgene expression of human IL-1Ra, and this experiment required a 10^3 higher MOI to combat an increase in PGE_2 production by synoviocytes 12 hours after exposure to IL-1β.[132] Difference in IL-1 molecules, sampling interval, or transgene expression may have accounted for some differences between the results of the two studies. The concentration of IL-1α used in the current study was based on a dose in the plateau region of a dose response curve (data not shown). Furthermore, both studies utilized adenoviral vectors; however the normalization of IL-1Ra levels differed in the two studies prohibiting further comparisons.

Based on the results of the *in vitro* studies, the outlined specific aims had been completed and further testing of the vector *in vivo* was undertaken.

2.4.2 In vivo

A dose response was seen in synovial fluid IL-1Ra levels with increasing concentrations of AdEqIL-1Ra vector administration (Figure 7). Peak levels were observed at the highest concentration of vector, however, this concentration was also associated with the shortest measured transgene expression period. IL-1Ra concentrations in the current study were similar to those previously reported in other gene transfer protocols where beneficial effects of IL-1Ra expression were demonstrated on joint tissues in experimental models of joint disease.[19, 24, 25, 132, 141, 159] The duration of detectable IL-1Ra levels were 3 – 4 times longer than those seen with other adenoviral vectors.[132] Differences in viral preparation, permissiveness of cells to transduction or reaction to viral protein products are likely responsible for these findings. However, the current study is novel in the use of a gene sequence

derived from the same species intended for transgene expression and may also contribute to the observed results. Previous literature has cited retroviral vectors associated with longer transgene expression periods due to stable integration of the transgene into the host cell genome as well as less viral protein expression potentially inciting less immunogenic response.[34, 62, 152, 160] However, transgene expression periods achieved in this study, using the Ad-EqIL-1Ra vector, are comparable to those demonstrated in previous reports using retroviral vector systems.[19, 24, 25, 141, 159, 161] The production of increased IL-1Ra in a non-transduced joint is interesting, especially after the IL-1Ra levels in the synovial fluid would suggest transgene expression had ceased (Figure 8). These results may be representative of a positive feed back loop inducing endogenous IL-1Ra gene expression or may be a return of transgene expression after loss of an inhibitory stimuli. The increased IL-1Ra levels in the placebo treated joint would favor an endogenous upregulation of IL-1Ra production. Recent reports of leukocytes carrying transgenes trafficking to remote joints could also explain these results. A definitive answer is beyond the scope of the study, given tissue or another source of mRNA was not available to differentiate transgene versus endogenous gene expression. However, this phenomenon will be screened for in future studies.

The highest concentration of vector produced the greatest inflammatory reaction as measured by WBC influx into the synovial fluid (Figure 9). This increase in cell numbers occurred proportionately to the normal differential cell population, meaning the increase was not one specific type of WBC. It is well accepted that adenoviruses evoke direct cellular pathology, as well as both cellular and humoral immune responses. Many of the responses categorized to date are mediated by T-cells.[61-64, 162-165] At similar adenoviral vector concentrations, cellular responses comparable to those observed in the current study have been demonstrated.[165] An unexpected finding was the

increase in neutrophil numbers in synovial fluid from joints transduced with either 0.1 or 1.0×10^{10} particles/joint. The viral stock used in these transductions was different than those used at the remaining tested concentrations. Because the neutrophilic response was only seen at lower concentrations of vector, and did not increase with higher concentrations, (Figure 10) this response was most likely due to a lower purity level of viral stock.

The *in vitro* studies described in this manuscript support that the Ad-EqIL-1Ra vector is expressing the IL-1Ra transgene and secreting an active protein. Furthermore, based on the *in vivo* results a dose of 20×10^{10} particles/joint was chosen for future studies based on the level of IL-1Ra achieved and lack of a cellular response, indicating nominal immune stimulus.

CHAPTER 3

3 *In vivo* gene transfer of IL-1Ra using an adenoviral vector in an osteochondral fragment model of joint disease in exercised horses.

3.1 Introduction

Diseases of the musculoskeletal system are among the most common equine afflictions. In one survey, 19% of all equine patients had lameness related to their presenting complaint and 42% of these lame equine patients had lameness directly related to joint disease.[1] Osteoarthritis (OA) has been specifically cited as the most economically important musculoskeletal disease in both athletic and pleasure horses.[5, 6]

Numerous conventional therapeutics in joint disease have been utilized to manage the pathologic manifestations of equine joint disease. To date, these treatments have been directed towards lowering and then maintaining a decreased degree of inflammation within damaged joints.[8] These treatments have consisted of nonsteroidal anti-inflammatory drugs (NSAIDs) and corticosteroids, using their anti-inflammatory properties as the mode of treatment. Although symptomatic relief may be obtained with these agents, little protection is afforded to the articular cartilage, nor do these drugs modify the underlying disease process. Both agents have also been associated with undesirable side effects.[8, 9]

Therapeutic intervention in OA is hindered in part by the inability to target therapeutic agents directly to the joint. Traditional routes of administration can be relatively ineffective at delivering therapeutic levels of agents to the joint space. Although intraarticular (IA) administration avoids these limitations, the half-life of most commonly used agents administered directly into the joint space is short,[17, 18] and frequent IA injections are needed to sustain biologic activity for prolonged treatment of chronic diseases. In addition, many drugs cannot be used by direct IA injection. In an attempt to circumvent some of these limitations, gene transfer is being investigated to deliver proteins of therapeutic benefit to the joint space.

Gene transfer provides an excellent alternative to conventional therapy whereby a single IA injection can result in local production of a specific therapeutic protein within diseased joint(s) for a prolonged period of time.[19] In rabbits, IA expression of IRAP (driven by retroviral vector transduction) has been demonstrated to provide marked chondroprotective effects (decreased glycosaminoglycan degradation, maintenance of GAG synthesis, anti-inflammatory effects, decreased leukocyte count in synovial fluid) in an antigen-induced model of OA.[19] A canine *in vivo* (Pond-Nuki) model of OA has also shown the ability of gene transfer using IRAP to decrease joint tissue pathology reducing both macroscopic and histologic articular cartilage lesions in the stifle.[141] These studies have sparked great interest in IRAP expression using gene transfer in other species with naturally occurring joint disease, such as the horse.

Recently the quantification of mRNA levels has provided insight into the possible roles specific molecules may play in the cascade of events occurring in pathologic joint tissues. This study, in addition to evaluating the potential beneficial effects of IL-1Ra, will also evaluate the expression of mRNA molecules known to be important in the pathology of joint disease. IL-1 is one such mediator, and has been established as a major cytokine in joint disease.[81, 166-171] TNF-α has similar biologic actions to IL-1

but has been shown to act on a separate cell membrane receptor.[172] IL-1 and TNF-α have also been suggested by some to have similar and synergistic effects.[172-174] Both cytokines show catabolic and suppressed anabolic effects on articular cartilage matrix that are detrimental to the health of articular cartilage. Production of both cytokines by chondrocytes and synoviocytes has been demonstrated.[175, 176] Finally, similar effects of both cytokines have been demonstrated on equine chondrocyte matrix metabolism, as has been observed in human chondrocytes *in vitro*, suggesting similar actions of IL-1 between the two species.[94, 95, 102] Collectively these properties make the gene products of IL-1 and TNF-α suitable for monitoring in these studies.

Metalloproteinases are also considered to play a major role in the catabolic metabolism of the articular cartilage matrix.[8, 177, 178] The stimulation of these products is thought to be largely mediated by IL-1 and TNF-α in chondrocytes and synoviocytes.[179, 180] Of note, stromelysin and collagenase have been shown to be active in OA and RA patients[181-183] and to be at least partially responsible for the destruction of proteoglycans and type II collagen respectively.[184-186] Recent evidence that MMP-13 plays an active role in joint pathology also warrants its inclusion in the mRNA evaluation.[187, 188]

The inclusion of IL-1Ra in mRNA quantification will potentially shed light on this molecule's role in relation to the other quantified mediators. Additionally a sequence specific for IL-1Ra produced from the transgene will be quantified to assess the presence of transgene product 54 days after transduction.

The specific aims of this study were to measure the therapeutic effects of IL-1Ra on a traumatic and inflammatory model of joint disease in the horse using clinical pathology, histology, histochemical, biochemical and molecular biologic techniques.

3.2 Materials and methods

3.2.1 Experimental design

Sixteen skeletally mature horses, aged 2-5 years, were used in the study. Horses were in good health, without palpable carpal effusions, and free of lameness before and after carpal manipulation. Complete radiographic examinations of the carpi were performed on each horse. Each horse was acclimated (2 - 3 training sessions) to a high-speed treadmill for a 2-minute trot (8-12 mph), 2-minute gallop (25-33 mph) and 2-minute trot protocol to simulate athletic race training.

The horses were divided into two groups (treated and placebo). All horses had an osteochondral fragment (chip) created in one randomly selected intercarpal joint, and the opposite joint served as the control. During the surgical procedure, synovium was harvested for mRNA analysis. Intra-articular treatment with an adenoviral vector carrying the equine IL-1Ra sequence occurred 14 days after surgery (chip creation). The "treated" group had 20 x 10^{10} Ad-EqIL-1Ra viral particles/joint diluted to a total volume of 1ml with Gey's balanced salt solution (GBSS), administered IA into the joint containing the chip, and the opposite joint (control) received a similar volume of GBSS. The "placebo" group received IA administration of 1ml GBSS in both intercarpal joints.

3.2.2 Fragment creation

Briefly, the osteochondral fragments (chips) were created as follows. Each horse was medicated with procaine penicillin (22,000 IU/kg, intramuscularly for 3 days) and phenylbutazone (4.4 mg/kg, orally for 3 days) starting before surgery. Each horse was premedicated with xylazine, anesthetized with guaifenesin and thiopental, and maintained with halothane in 100% oxygen through a semi-closed breathing system. Routine bilateral carpal arthroscopic surgery was performed after sterile preparation and

joint fluid aspiration.[189] One milliliter of joint fluid was placed into a tube containing ethylenediaminetetraacetic acid (EDTA) for determination of color, clarity, mucin content, total protein and inflammatory cell concentrations. The remainder of the synovial fluid was placed into a sodium citrate tube, centrifuged at 1,000g for 30 minutes, and the supernatant was stored at -80C.

A lateral arthroscopic portal was used in all joints and an instrument portal was placed medial to the extensor carpi radialis tendon at the level of the third carpal bone in one randomly chosen joint of each horse. This portal allowed an 8 mm curved osteotome to be directed perpendicular to the articular cartilage surface of the radial carpal bone at the level of the medial synovial plica. An osteochondral fragment was created with the osteotome, and the fragment was allowed to remain adherent to the joint capsule proximally. Exposed subchondral bone between fragment and parent bone was debrided using a motorized arthroburr to form a 15mm wide defect bed for the 8mm wide fragment (Figure 12).[153, 154] Diagnostic arthroscopy was performed on the contralateral intercarpal joint to ensure absence of any joint problems. All arthroscopic portals were closed using 2-0 nylon in a cruciate pattern, the limbs were bandaged and the horses were recovered. After surgery, all horses were kept in 3.65m by 3.65m stalls. Bandages were changed every 3 to 5 days and maintained for 14 days, and sutures were removed 10 days after surgery.

3.2.3 Exercise protocol

Horses were housed in stalls unless otherwise noted. Exercise on a high speed treadmill began on day 14 post fragment creation and continued 5 days per week until day 70. The treadmill exercise protocol consisted of a 2-minute trot (8-12 mph), 2-minute gallop (25-33 mph) and 2-minute trot.

3.2.4 Clinical examinations

Lameness examinations on day 70 were performed on all horses at a trot and baseline lameness graded 0 to 5 according to guidelines established by the American Association of Equine Practitioners.[155] Carpal flexion was also performed for 45 seconds, after which time the horses trot in a straight line, and the response to flexion of the carpus was graded on a scale of 0 to 4 (0 = no response, 1 = slight response, 2 = mild response, 3 = moderate response, and 4 = severe response). Synovial fluid effusion was also graded subjectively as having normal, slight, mild, moderate, or severe synovial effusion. This grade was converted to a numeric value (0 – 4), with 0 being normal and 4 severe, and used in the statistical analysis.

Radiographic examinations (anterior – posterior and flexed lateral) were performed at Day 0 and 70 post-chip creation of both intercarpal joints. Radiographs were graded by a radiologist unaware of the treatment assignments, for changes in the subchondral bone and for periarticular osteophyte formation. A grading scale 0 - 3 was utilized to statistically evaluate radiographic changes (0 = no significant changes, 1 = focal subchondral bone lysis on either view, 2 = diffuse, shallow subchondral bone lysis, 3 = deep subchondral bone lysis with or without bone fragmentation).

3.2.5 Synovial fluid analysis

Synovial fluid was collected at the time of surgery and 9 additional evenly spaced time periods between surgery and the termination of the experiment, 70 days after surgery. Synovial fluid color, clarity and mucin content were evaluated subjectively, and total protein and inflammatory cell (WBC) concentrations were determined by use of routine clinicopathologic methods.[iii] Color of synovial fluid was graded as yellow, colorless, straw, orange or red and numeric values of 1 – 5, respectively, were assigned. Clarity was graded as clear or cloudy and numeric values 1 or 2, respectively, were

assigned. Mucin content was graded as good, fair or poor and numeric values of 1 – 3, respectively, were assigned.

All chemical reagents unless otherwise indicated were obtained from one commercial source. Concentrations of IL-1Ra and PGE_2 were estimated from collected media stored at –80 C. Neat media aliquots were used for determination of IL-1Ra utilizing a commercially available kit and manufacturer's recommendation.[i] Determination of PGE_2 was performed using a commercially available kit and manufacturer's recommendation for the analysis of synovial fluid.[ii]

3.2.6 Postmortem examination

The horses were euthanized with an overdose of sodium pentobarbital in accordance with Colorado State University Animal Care and Use Committee (ACUC) guidelines. Each intercarpal was aseptically prepared and opened for harvest of cartilage and synovial membrane. Photographic documentation of joint tissue pathology was then performed, and the extent of articular cartilage erosions as well as the bone on which they occured was also recorded as none, slight, mild, moderate or severe (numeric values assigned 0 – 4, respectively). Any significant other abnormalities in the joint tissue were also recorded.

3.2.7 Histologic evaluation of synovial membrane

Synovial membrane and joint capsule were harvested just dorsal to the osteochondral fragment, and placed in 10% buffered formalin for hematoxylin and eosin staining (H & E). Five micron sections were evaluated and graded blindly for cellular infiltration, synovial intimal hyperplasia, subintimal edema, subintimal fibrosis and vascularity.[153, 154] Each variable was graded and reported 0 to 4 (0 = normal, 1 = slight change, 2 = mild change, 3 = moderate change, and 4 = severe changes).

3.2.8 Histologic and histochemical evaluation of articular cartilage

Articular cartilage pieces, 5 mm^2, were obtained from the radial and 3rd carpal bones, as well as the 4th carpal bone (Figure 12). These were stored in 10% buffered formalin for 7 days. Half of the 5 μm sections obtained were stained with H & E, and the remainder with safranin-O fast green (SOFG).

Sections stained with H & E were evaluated blindly for articular cartilage fibrillation, chondrocyte necrosis, chondrone formation and focal loss of cells.[153, 154] Cartilage fibrillation was scored 0 for no fibrillation, 1 for surface fibrillation, 2 for clefts into the tangential zone, 3 for clefts into the radiate zone, or 4 for clefts into the calcified cartilage layer. Chondrocyte necrosis, chondrone formation, and focal cell loss was each graded 0 to 4 for the severity of change in each parameter (0 = normal, 1 = slight change, 2 = mild change, 3 = moderate change, and 4 = severe changes). Total scores was determined and reported for each section with a total possible score of 14.

Articular cartilage sections stained with SOFG were evaluated blindly for intensity of staining in the tangential, intermediate, radiate territorial and radiate interterritorial zones.[153, 154] The intensity of stain uptake was graded and reported 0 to 4 for each section (0 = no stain uptake, 1 = slight stain uptake, 2 = mild stain uptake, 3 = moderate stain uptake, 4 = normal stain uptake).

3.2.9 Articular cartilage matrix evaluation

To estimate articular cartilage proteoglycan content the total articular cartilage glycosaminoglycan (GAG) content was measured. Briefly, articular cartilage pieces (80 to 100 mg wet weight), were obtained aseptically from the osteochondral fragment, the radial and intermediate carpal bones (Figure 12). Until further processing, each piece was stored at -80C prior to analysis. Total GAG content for each piece was determined by the 1,9-dimethyl methylene blue dye binding method.[190] The assay was

standardized with shark chondroitin sulfate in de-ionized distilled water. Samples were run in duplicate, and samples reported as μg GAG / mg cartilage dry weight (dw).

For analysis of cartilage matrix metabolism, articular cartilage samples were aseptically collected from the weight-bearing surfaces of the intermediate carpal bones. The pieces (weighing 80 to 100 mg wet weight) were combined and placed in Gey's balanced salt solution on ice until further processing. The samples were then placed into Dulbecco's Modified Eagles Medium with additives, and incubated for 2 hours at 37C in 5% CO_2. Each piece was then incubated with 20 μCi / ml $^{35}SO_4$ in media for 16 hours. After the incubation period, each piece of articular cartilage was stored at -80C and all samples were batched for analysis. All articular cartilage pieces were subsequently freeze-dried for 48 hours and dry weights determined. Each piece was digested in papain solution (2X papain, 0.05 M phosphate buffer, 2 mM N-acetyl cysteine and 2 mM EDTA) at 65C for 4 hours to solublize the proteoglycan. Using previously reported methods PG synthesis was estimated (alcian blue modified dye-binding technique).[191] Samples were run in duplicate and the results reported as counts per minute (CPM) / mg dw.

3.2.10 Semi-quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) evaluation

The mRNA expression of certain molecules known to be mediators of joint disease were quantified using qRT-PCR in tissue collected from individual joints. Specific amplimers representing MMP-1, MMP-3, MMP-13, IL-Ra, vector derived IL-1Ra (vIL-1Ra), IL-1α, IL-1β, and TNFα, TNFα receptors type I and II (TNFrI and TNFrII, respectfully) mRNA were examined. Primers were also designed to amplify cDNA derived from the IL-1 receptors, however, the primers did not function as expected and were therefore omitted. Specific primer sequences used in the qRT-PCR evaluation can

be found in Table 2. During the arthroscopic surgery (day 0) a synovial biopsy was obtained from each joint as well as during necropsies performed on day 70. Synovium was harvested from the synovial pad on the dorsal medial surface of the intercarpal joint (similar area on days 0 and 70 but different locations) and at the time of necropsy cartilage samples were obtained from the radial, ulnar and 3rd carpal bones (Figure 12). Samples were stored frozen (-80C) in Trizol[iv] until further processing.

The samples were thawed and homogenized using a Polytron homogenizer[v] for small tissue samples. The samples were then centrifuged at 8,000g for 5 – 10 min at 2 – 8C. The supernatant was removed and the homogenate was incubated at room temperature for 5 minutes to allow complete dissociation of the nucleoprotein complexes. Phase separation was accomplished by adding chloroform (0.2μl/ml Trizol) and mixing thoroughly followed by a 2 – 3 min incubation at room temperature (RT). The samples were centrifuged at 12,000g for 15 min at 2 – 8C and the RNA in the aqueous phase was transferred to a new tube. Isopropanol (0.5ml/ ml of aqueous phase) was then added to precipitate the RNA. The sample was incubated at 15 – 30C for 10 min followed by centrifugation at 15,000g for 30 – 45min at 2 – 8C. The RNA pellet was then evaluated for either protein or for low yield of RNA. If contamination was present, the pellet was re-suspended in 4M LiCl to differentially solubilize the proteins and RNA. If RNA yield was low, 10μg of glycogen was added and precipitation was repeated overnight at –80C. A visible pellet was obtained upon subsequent centrifugation. The supernatant was removed and the RNA pellet was washed in 1ml of 75% ethanol/ ml Trizol reagent. Centrifugation was then performed at 16,000g for 15 min at 2 – 8C. Following removal of the supernatant, the RNA pellet was dried for 3 minutes at 65C. The RNA was then dissolved using RNAse free water and incubated for 10 min at 55 – 60C. Total RNA recovered was quantified by determining its optical

density (OD) at a wavelength of 260nm (OD_{260}) with a Beckman DV640 spectrophotometer. RNA quality was assessed by the OD_{260}/OD_{280} ratio.[151]

The samples were then subjected to reverse transcription using a calculated quantity of RNA to obtain 1μg of DNA in 10.5μl of diethyl pyrocarbonate (DEPC) treated water. The RNA was added to a mixture containing 2μl of 25mM $MgCl_2$, 2μl of 10mM dNTPs, 0.5μl RNAse inhibitor, 2μl 10X PCR buffer with $MgCl_2$, 2μl random hexamer primers, and 1μl Moloney Murine Leukemia Virus reverse transcriptase.[vi] This mixture was incubated at 37C for 1hr. Two microliters of the above cDNA mixture was then combined with 8μl of the following PCR master mix: 10μl upstream primers, 10μl downstream primers, 8μl 10X PCR buffer with $MgCl_2$, 51.5μl DEPC treated water, 1μl Triton X-100, and 0.5μl *Taq* DNA polymerase.[vi] The cDNA mixture was processed in a thermocycler at the appropriate annealing temperature to allow for annealing and extension of the primers. The number of cycles completed depended on the original amount of RNA present in each sample. If the sample contained 1μg of RNA, then 30 cycles were performed; any sample containing less than 1μg of RNA was subjected to 55 cycles. The cDNA samples were stored at –20C prior to further analysis. Application of mRNA GAPDH was performed on each sample as an internal control as all samples that had adequate GAPDH mRNA present were considered to have adequate RNA present to be able to amplify specific sequences. Equine specific primers were utilized for MMP-1, MMP-3,[vii] MMP-13,[viii] IL-1α, IL-1β, IL-1Ra, vIL-1Ra, and TNFα. Degenerate primers were designed based on comparison of human and other laboratory species gene sequences for both the type I and II TNF receptors. PCR products were electrophoresed on a 3.5% Nusieve agarose gel, and intensity of ethidium bromide stained bands were quantified comparing the sample to the internal control of GAPDH. Signal intensity of the band for each sample was normalized with a known concentration

produced from the molecular weight marker (ϕX174/*Hae* III)[vi] and GAPDH signal for each sample. Results were expressed as a ratio of ng for specific cDNA to ng DNA specific for GAPDH.

3.2.11 Statistical analysis

Dependent variables that were measured at more than two time points were analyzed in the following manner. A mixed model analysis of variance (ANOVA) was performed using Proc Mixed. Independent variables appearing in the model statement were day sample collection occurred post-chip creation, presence or absence of a chip in the joint/limb, and treatment, measuring if the horse was treated with placebo or Ad-EqIL-1Ra. The subject within treatment variables, as well as, the interaction between chip and subject horse within treatment variables were used as random effect variables. The time (day of collection) variable was also utilized in the repeated statement with the interaction between and subject within treatment variables acting as the subject, using a type I auto-regressive covariance matrix. Dependant variables measured at 2 or less time points had a similar analysis performed. However, the model statement only utilized the presence of chip, treatment and the interaction between these variables, and the random statement only utilized the subject within treatment variables. When individual comparisons were made, a Least Squares Means was utilized.

3.3 Results

3.3.1 Clinical examinations

Throughout the duration of the study no horse was observed with a lameness that prohibited continued exercise. Furthermore the highest degree of lameness was a grade 3 in one horse and all other horses were either grade 1 or 2 in any limb.

Analyzing lameness examinations performed 70 days post-chip creation (54 days after treatment) demonstrated that limbs of placebo treated horses containing chipped joints had a higher lameness score compared to limbs with non-chipped joints (Figure 13). No statistical difference was noted between limbs containing either chipped or non-chipped joints in the Ad-EqIL-1Ra treated horses, however, limbs that contained chipped joints that were treated had significantly lower lameness scores as compared to limbs containing chipped joints in placebo treated horses (Figure 13). On day 70 after chip creation a higher synovial effusion score was observed in joints with a chip compared to non-chipped joints, in both placebo and Ad-EqIL-1Ra treated horses. Joints treated with Ad-EqIL-1Ra and containing a chip, demonstrated significantly lower synovial effusion scores compared to the chipped joint and no difference compared to the non-chipped joint of placebo treated horses (Figure 14), indicating an improved synovial effusion score with Ad-EqIL-1Ra treatment in chipped joints. No significant difference in the response to carpal flexion was noted between any comparison 70 days after osteochondral fragmentation.

The evaluation of the carpal radiographs indicated no significant lesions in non-chipped joints of either treated or placebo horses. Chipped joints did have significantly higher radiographic lesion scores as compared to non-chipped joints form all horses (1.8 ± 0.1 and 0.0 ± 0.1, respectively). Although no significant difference was seen when comparing chipped joints form treated versus placebo horses (1.7 ± 0.2 and 1.9 ± 0.2, respectively).

3.3.2 Synovial fluid analysis

No significant changes in the synovial fluid color or mucin clot scores were seen or any comparisons made. Synovial fluid scores for clarity were affected by treatment, independent of presence or absence of a chip or day of sample collection. An increase

in these scores was observed after administration of the Ad-EqIL-1Ra vector, returning to baseline levels by the conclusion of the study; the average clarity score (mean ± standard error) from joints of treated versus placebo horses were 1.24 ± 0.03 and 1.15 ± 0.03, respectively. The synovial fluid concentration of total protein was elevated in association with the collection period, as well as either presence or absence of a chip and Ad-Eq-IL-1Ra vector or placebo treatment. All chipped joints experienced a significant increase in synovial total protein concentrations compared to non-chipped joints, 3.02 ± 0.08 and 2.56 ± 0.08 (P – value 0.0001) respectively. Ad-EqIL-1Ra joints, especially Ad-EqIL-1Ra treated chipped joints, had higher values as compared to similar (chipped versus non-chipped) placebo treated joints. However, by day 70 post-chip creation, synovial fluid total protein levels were significantly lower in treated non-chipped joints as compared to all other joints at this time period. Although Ad-EqIL-1Ra treated chipped joints had a lower mean compared to placebo treated joints, this difference was not statistically different (Figure 15). The concentration of WBCs measured in Ad-EqIL-1Ra treated chipped joints was significantly higher on days 21 and 28 post-chip creation (7 and 14 days post Ad-EqIL-1Ra treatment) compared to all other joints at any time. However, the highest (day 28) average WBC concentration was 1,862 ± 204 cells/μl and returned to baseline levels by day 35 post-chip creation. The specific population of cells in the synovial fluid differed based on whether the synovial fluid was from Ad-EqIL-1Ra or placebo treated horses and the time period of the sample post-chip creation (P – value 0.0005). Ad-EqIL-1Ra treated horses had higher percentages of monocytes in their synovial fluid on day 21 and lower percentages on day 28 post-chip creation compared to placebo treated horses (Figure 16). The mirror image of this change was seen in the percentage of lymphocytes within the synovial fluid for the same comparisons (data not shown).

IL-1Ra concentrations were significantly increased in chipped joints transduced with Ad-EqIL-1Ra on days 21, 28 and 35 post-chip creation (7, 14 and 21 days post transduction) (Figure 17). A trend for a small increase in IL-1Ra concentrations was also noted at day 63 post-chip creation although it was not statistically significant.

PGE_2 concentrations were significantly increased in all joints 7 days after arthroscopy and in joints containing a chip compared to non-chipped joints after day 7 (Figure 18). Even though on average over the entire study period non-chipped joints had significantly higher synovial fluid PGE_2 levels as compared to chipped joints no significant differences in treated or non-treated chipped joints were observed, a trend did exist for lower PGE_2 levels in chipped joints of treated verses placebo horses.

3.3.3 Postmortem examination

All intercarpal joints contained some level of pathologic change in the form of partial or full-thickness articular cartilage erosions, mostly on the 3rd carpal bone. The most dramatic changes were noted in chipped joints from placebo treated horses. Not only were there full-thickness erosions observed secondary to kissing lesions, but 3rd carpal bone full thickness erosions were also observed independent of the location of the fragment. Similar lesions on the 3rd carpal bone were observed in chipped joints treated with Ad-EqIL-1Ra, however, the remaining joint surfaces had fewer significant lesions (Figures 19 & 20). Adhesions of the synovial membrane in the area of osteochondral fragmentation were noted in two joints from placebo treated horses. No other significant abnormalities were identified.

3.3.4 Histologic evaluation of synovial membrane

The scores for cellular infiltration were significantly higher in joints containing a chip compared to those that did not (P- value 0.0004). Furthermore, joints treated with

Ad-EqIL-1Ra and containing a chip had significantly higher scores than any other joints (Figure 21). The cellular infiltration was primarily characterized by perivascular lymphocytic infiltration (Figure 22). Joints containing a chip had significantly more intimal hyperplasia compared to non-chipped joints (P – value 0.0498), and although the administration of Ad-EqIL-1Ra lessened these scores, this difference was not statistically different (P – value 0.1546). The scores for subintimal edema were significantly higher for chipped joints (1.9 ± 0.4) compared to non-chipped joints (0.9 ± 0.4) from horses receiving Ad-EqIL-1Ra, and although they were higher than those from chipped joints of placebo treated horses (0.8 ± 0.4) the difference was not statistically different (P – value 0.0762). No significant differences were noted for subintimal fibrosis in any comparison, but all joints from Ad-EqIL-1Ra treated horses had significantly lower vascularity scores compared to those from placebo treated horses (Figure 22 & 23).

3.3.5 Histologic and histochemical evaluation of articular cartilage

Five micron articular sections were evaluated from 3 different locations within each joint for fibrillation, chondrocyte necrosis, chondrone formation, and focal cell loss (Figure 12). Because similar results were seen from each location the results for each category were totaled for all sections. The score for chondrone formation was significantly higher from joints that contained a chip independent of treatment (P – value 0.0506) compared to non-chipped joints; no other categories had statistically significant findings.

Articular cartilage sections stained with SOFG were also evaluated from 3 different areas within each joint (Figure 12). Four staining patterns were evaluated on each section (see Materials and Methods) and similar results were obtained from all locations within the joint, as well as for the 4 staining patterns. Therefore, for ease of reporting a total score for SOFG staining was determined (0 – 48 possible score). Using

this score it was demonstrated that articular cartilage from chipped joints of placebo treated horse had significantly less SOFG staining than articular cartilage from any other joints (Figure 24 and Figure 25).

3.3.6 Articular cartilage matrix evaluation

Two different articular cartilage locations were harvested for DMMB assay to use as an estimation of articular cartilage proteoglycan content (Figure 12). The lowest values were seen in articular cartilage harvested from chipped joints of placebo treated horses, and the highest from non-chipped joints of placebo treated horses. Values for cartilage harvested from Ad-EqIL-1Ra treated joints tended to have higher values as compared to placebo treated joints, however, no significant differences were noted for any comparisons.

Articular cartilage was harvested from one location to evaluate proteoglycan synthesis at day 70 post-chip creation. Results of the alcian blue modified-dye binding assay indicated a significantly higher proteoglycan synthesis overall in joints containing a chip (independent of treatment) (3427 ± 360 CPM/mg dw) as compared to non-chipped joints (2541 ± 369 CPM/mg dw, P – value 0.0397). Specifically comparing Ad-EqIL-1Ra (3823 ± 522 CPM/mg dw) and placebo treated (3030 ± 522 CPM/mg dw) chipped joints, there was a higher proteoglycan synthesis in Ad-EqIL-1Ra treated joints, although this difference was not statistically different (P – value 0.2944).

3.3.7 qRT-PCR evaluation

Results of the ANOVA analyses using synovium or articular cartilage samples did not show any significant difference between presence or absence of a chip, or between placebo or Ad-EqIL-1Ra treatment. However, significant increases in expression of mRNA for MMP-3, IL-1Ra, IL-1α, IL-1β, TNFα, TNFrI, and TNFrII were observed from

Day 0 compared to Day 70 post-chip creation. Furthermore, MMP-13, TNFrI or TNFrII were not detected in synovium at any time point and MMP-3, IL-1Ra, IL-1α, IL-1β, and TNFα were expressed in the highest frequencies on day 70 post chip creation (Table 3). In articular cartilage, MMP-1 was the only amplimer that was not detected and MMP-3, IL-1β, TNFα, TNFrI and TNFrII were expressed in the highest frequencies on day 70 post-chip creation (Table 4). Interestingly IL-1Ra sequence specific for vector mRNA was detected 54 days post vector administration in one horse from both cartilage and synovium.

3.4 Discussion

A number of studies have been conducted to assess IL-1Ra administration on normal and diseased joint tissues using both experimental and clinical settings.[19, 24, 25, 28, 133-138, 140, 141, 192, 193] Except for one study,[193] beneficial effects on joint tissues were shown. To circumvent the need for repeated protein administration, some of these studies utilized gene transfer to obtain sustained IL-1Ra with one procedure.[19, 24, 25. 28, 137, 140, 141, 192] However, with the exception of Pelletier, et al.[141] controlled experimental studies were completed in species not typically recognized or treated with naturally occurring joint disease. Furthermore, with the exception of the work completed by Evans and colleagues,[28] human gene sequences have been expressed in a non-human species adding additional variables to the interpretation of the results. The study presented here not only utilized a species with naturally occurring joint disease, but the IL-1Ra gene sequence was expressed from the species from which it was obtained.

Clinical examinations of the horses indicated that the therapeutic expression of IL-1Ra significantly decreased signs of joint pain as measured by degree of lameness. The amount of synovial effusion associated with the chip was also significantly

decreased in joints administered Ad-EqIL-1Ra. Although a relatively non-specific evaluation of joint pathology, the degree of synovial effusion is often a good indicator of disease activity.[1, 146, 194] These improved clinical findings are similar to those observed by others[131, 140, 195] and are essential in the therapy of naturally occurring joint disease in any species. Although decreased radiographic joint degeneration has been associated with IL-1Ra administration[138] the current study while observing similar trends did not see a statistically significant difference. The current study did have a 16 week shorter experimental period, which may have contributed to the lack of significant results.

Significant changes in synovial fluid parameters were seen in association with Ad-EqIL-1Ra administration. Specifically an increase in the synovial fluid total protein concentration was observed in response to creation of a chip, as well as Ad-EqIL-1 administration. Higher total protein levels in joints that contain a chip have been previously observed in both a similar experimental model, as well as in naturally occurring cases of osteochondral fragmentation.[153, 154, 196] The increase in synovial fluid total protein levels specifically associated with Ad-EqIL-1Ra administration is most likely due to a combination of increased IL-1Ra production, and immunogenic response to the vector and other vector gene products. This increase in synovial fluid total protein has not been previously reported, most likely in part due to the size of the synovial cavity from the experimental subjects prohibiting its measurement. Synovial fluid volume constraints in many other species have lead to the incomplete evaluation of the synovial fluid constituents in response to treatment. Historically parameters that were evaluated have been limited to extrapolations for inflammatory cell and IL-1Ra concentrations. Further defining the specific protein fraction that was increased in our results was beyond the scope of this study. Classically, in naturally occurring joint disease a lower synovial fluid total protein concentration has been associated with less

joint pathology,[146, 197] and the lower levels found in the synovial fluid from Ad-EqIL-1Ra treated joints may therefore be of benefit.

A clear cellular response to the direct IA administration of Ad-EqIL-1Ra was measured in the synovial fluid through WBC concentrations. The levels were not different than those observed in the *in vivo* dose titration of this vector, and the absolute values observed in this study would be considered near normal (1,670 cells/dL) and were well below that of idiopathic synovitis.[196] Interestingly the highest WBC concentrations were 14 days after vector administration (day 28 post –chip) and did not appear to be correlated with IL-1Ra concentration (data not shown). Analysis of the specific cell types comprising the WBC count demonstrated an initial increase in mononuclear cells with a parallel decrease in lymphocyte numbers 7 days after vector administration. A complete reversal of these findings occurred by the next sampling period (day 14 after vector administration) and this was followed by a slow return to baseline levels for both cell types through day 49 post-chip creation (Figure 16). Others specifically evaluating synovial membrane response to IA adenoviral gene transfer have found very similar results.[163] This report observed a peak inflammatory response 7 days after vector administration. Lymphocytes predominated in the chronic phases prior to resolution of the inflammation by day 21 post vector exposure. The use of anti-T-cell receptor antibodies completely blocked the lymphocyte response and prolonged transgene expression. Although the inflammatory response seen in the current study was mild, it is likely that T-cell mediated destruction of transduced cells was partially responsible for transient transgene expression and methods of modulating this immune reaction may be beneficial in future studies.

Synovial fluid IL-1Ra levels were significantly elevated in joints 7 days post-AdEqIL-1Ra vector administration (peak concentration 19,947 ± 1243 ρg/ml synovial fluid). IL-1Ra levels were higher than placebo treated joints until 28 days post vector

administration (Figure 17). Furthermore, IL-1Ra concentrations in the current study were similar to those previously reported in other gene transfer protocols showing beneficial effects of IL-1Ra expression on experimentally induced joint disease.[19, 24, 25, 132, 141, 159] The duration of transgene expression appeared to be similar between the current study and the previous studies performed by the author (Chapter 2). However, in the previous study the average peak IL-1Ra concentration was lower at a similar vector dose (Figure 7). Differences in the viral stocks, transduction frequencies, or transgene expression variables in normal verses chip joints may account for the disparity in peak IL-1Ra concentrations. Definitively delineating these differences was beyond the scope of this study. However, IL-1Ra concentration and duration demonstrated in the current study are compatible with previous reports using retroviral vector systems.[19, 24, 25, 141, 159, 161] Furthermore, the apparent duration of transgene expression is significantly longer than other studies utilizing *in vivo* IL-1Ra gene transfer with adenoviral systems.[192] Loss of transgene expression is most likely due to adenoviral specific factors since the production of anti-IL-1Ra antibodies is unlikely to have occurred, given the use of the equine IL-1Ra gene sequence.

Similar to previous reports,[154, 198] PGE_2 levels in synovial fluids were elevated in response to surgery (all joints) and were further elevated significantly in joints where a chip was created. Other intra-articular therapeutics, such as sodium hyaluronate[198] and methylprednisolone,[154] have significantly lowered synovial fluid PGE_2 levels 56 days post administration and although a similar trend was seen in the current study it was not statistically significant. Other reports of IL-1Ra therapy have significant effects on articular cartilage and less consistent effects on inflammation often thought to be mediated through soft tissue of the joint may explain this finding.

Postmortem examinations indicated fewer gross pathologic changes existed in chipped joints administered Ad-EqIL-1Ra compared to joints from placebo treated

horses. Others have also reported less articular cartilage erosions associated with IL-1Ra administration both through direct IA administration of the protein[137] and gene transfer techniques.[141] In previous reports, IL-1Ra treatment was administered within 48 hours of the experimentally induced joint pathology, unlike the current study where treatment was not instituted until 14 days after induction of pathology, a time frame more consistent with clinical case presentation. These findings are very promising for long-term health of joints that have sustained trauma and are subsequently treated with IL-1Ra.

The administration of Ad-EqIL-1 was associated with synovial membrane perivascular lymphocytic infiltration and subintimal edema 54 days post-administration. The creation of a chip within the joint was also associated with similar changes but not to a similar extent. Although subintimal edema has not been described previously in association with IL-1Ra gene transfer, lymphocytic infiltration has.[19, 141, 163] In previous studies lymphocytic infiltration was resolved 21 days after vector administration and in the same study, shown to be neutralized by anti-T-cell therapy.[163-165] In another study marked cellular infiltration was still evident 4 weeks after vector administration.[141] The persistence of these finding in the current study are unknown, however, a difference between model chronicity may be a factor. It should be noted that even though the cellular infiltration and subintimal edema scores were abnormal, they were still graded as mild changes. On the other hand, the administration of Ad-EqIL-1Ra did have beneficial effects on the degree of synovial membrane vascularity in both joints of the Ad-EqIL-1Ra treated horses (Figure 23). An increase in synovial membrane vascularity has been the hallmark of active synovitis and improvement in this parameter could be beneficial.[146, 199, 200]

Articular cartilage sections obtained from chipped joints of placebo treated horses had significantly less SOFG staining as compared to all other evaluated sections

(Figure 24). This finding suggests that IL-1Ra had a protective effect on osteochondral fragment induced proteoglycan loss and is consistent with SOFG data observed by others.[141] Although analogous findings in the DMMB results were observed, the differences were not statistically significant. Previous reports have documented decreased proteoglycan degradation through the measurement of GAG concentrations in synovial fluid washes.[19, 132, 140] Because this methodology only measures total GAG content in the synovial fluid, the authors explanation of decreased degradation should be interpreted with caution, likewise making correlation to the current study difficult. Proteoglycan synthesis was higher in joints that contained a chip and the highest levels existed in Ad-EqIL-1Ra treated joints. However, without a statistically significant difference, further speculation is unwarranted.

The pathophysiology of joint disease as a whole is poorly understood, and less is known about the disease process specifically in the horse.[8, 146, 201] There have been many studies assessing the role specific mediators play in joint disease and these studies have often resulted in more questions than answers. However, the knowledge base continues to grow. In a similar attempt to build our knowledge base, synovial membrane and articular cartilage samples were analyzed using qRT-PCR for numerous mediators of joint disease (Table 2). From synovial membrane samples collected at day 0 positive amplimers for MMP-1, MMP-3, IL-1Ra, both forms of IL-1, and TNFα were demonstrated. With the exception of MMP-1 the number of positive amplimers obtained from day 70 synovial membrane was higher and interestingly mRNA from either form of the TNF receptors was not identified from synovium. When the ratio of specific mRNA to GAPDH was analyzed using an ANOVA, no statistically significant differences were noted between samples from either placebo versus Ad-EqIL-1Ra treated horses or non-chipped versus chipped joints. As expected significant increases were seen when comparing day 0 to day 70.

When specifically assessing the results from the articular cartilage samples no positive amplifications were observed at day 0, while at day 70 positive amplimers were seen for all amplimers with the exception of MMP-1. Results from articular cartilage samples after ANOVA analysis were similar to those seen from synovial membrane samples. Unlike synovial membrane, articular cartilage demonstrated positive amplimers for both forms of TNF receptors, suggesting a dominant expression from articular cartilage compared to synovial membrane. Interestingly vector specific mRNA for IL-1Ra was detected in both the synovial membrane and cartilage from the same joint of one horse at day 70, however no detectable protein was observed in the synovial fluid at a similar time point.

These results suggest that a controlled exercise protocol alone may be enough to induce mRNA production for many of the mediators examined in this study and further point out the tremendous need for appropriate control samples. It is important to note the results of the current study are not unique. Other reports using equine samples collected from clinical cases have observed similar findings.[202] The site of tissue collection with respect to focal disease and overall disease activity at sampled times in conjunction with the complex nature regulating these molecules most likely account for the results observed in both studies. Work assessing the effect of IL-1Ra protein administration on MMP-1 and MMP-3 mRNA levels in a Pond-Nuki model of OA in the dog showed significant reduction of cartilage MMP-1 mRNA levels using Northern hybridization.[137] Although both tested doses of IL-1Ra demonstrated a significant reduction in MMP-1 mRNA expression, levels were lower at the lowest tested dose (2mg versus 4mg OL-1Ra). In the current study only one sample had detectable MMP-1 mRNA. Similar to Caron, et al. no significant differences in MMP-3 mRNA levels for placebo compared to IL-1Ra treated joints were demonstrated.[137] *In vitro* studies using synovial tissue collected from human arthritic patients has also failed to

demonstrate correlation's between cytokine levels and the patients' medications or disease activity levels.[203] In a separate study, these authors demonstrated a very compartmentalized gene expression pattern using *in situ* hybridization, while also observing significant effects of corticosteroid administration on collagenase mRNA expression.[204] The potency of IL-1Ra compared to corticosteroids may be one explanation for the dissimilar results. It is also possible in the current study that effects of the treatment may have be masked by the sample collection methods, which were based on a specific location independent of pathology at a particular location. In future studies it may be beneficial to harvest tissue from specific locations and variable areas dependent on disease activity. A source of tissue that could be sampled throughout the induction and treatment of the pathology may also greatly enhance the results of this technique. Duration of time between the chip creation and day 70 sampling period may have also allowed time for normalization of the measured parameters, explaining the lack of significant differences. Finally, although many parameters of naturally occurring disease are reproduced using the described model, healing of the osteochondral fragment still occurs faster than it would in clinical cases. Suggests that this model, like most, is not perfect and significant differences in mRNA levels may reflect this.

In summary, this study was able to demonstrate an approximately 28 day effective upregulation of IL-1Ra through the use of gene transfer using an adenoviral vector. This increased level of IL-1Ra was associated with significant improvement in clinical parameters of pain and disease activity, as well as beneficial effects in histologic parameters measured from synovial membrane and articular cartilage. Similar to other studies using gene transfer techniques, results of this study suggest that reaction to vector related factors was responsible for limited transgene expression, emphasizing the need for improved engineered vectors that are less immunoreactive and potentially regulated. This study did, however, provide proof of principle that gene therapy using a

potentially anti-arthritic gene sequence is possible and efficacious in the horse and will hopefully serve as a cornerstone in future equine therapeutics.

4 Tables

Table 1 – Table indicating the transduction efficiency (%) of the AdLacZ vector on equine synoviocytes. The P- value associated with the comparison of various MOI concentrations compared to the O MOI group is shown.

MOI	Repetitions	Mean ± SEM	P – value
0	N=2	0 ± 4.1	N/A
10	N=2	21.7 ± 4.1	0.0195
100	N=2	66.1 ± 4.1	0.0003
500	N=2	91.3 ± 4.1	0.0001

Table 2 – Table defining the primer coding sequences for qRT-PCR evaluation, annealing temperatures and estimated amplimer length.

cDNA amplimer	Upstream primer (5' – 3')	Downstream primer (5' – 3')	Annealing Temp (C)	Estimated amplimer size (bp)
MMP-1	g^{a}/$_{g}$t gtg gg^{a}/$_{g}$ gti cc^{c}/$_{t}$ ga^{c}/$_{t}$ gtg	tac atc a^{a}/$_{g}$a gcc cc^{a}/$_{g}$ at^{a}/$_{g}$ tca	60	441
MMP-3	cat gga cct tct tca gga cta	ctccatgtt ctc gaa ctc c	60	420
MMP-13	ctt aga ggt gac tgg caa ac	ctg gta atg gca tca agg ga	62	657
IL-1Ra	cat cca gag act gcc tct cc	tta agt agg gcc gtg gtt tg	62	194
VIL-1Ra	aag ctt gca tgc ctg ca	att ctt gca act atc cag ca	58	200
IL-1α	acc aat gat gac ctg gaa gc	ttt aat gca gca gtc gca ag	58	200
IL-1β	cct cca aga cct gga cct ca	gcc aca atg att gac acg ac	60	108
TNFα	gat cat ctt ctc gaa ccc ca	tgt ccc ctg tct gtc ttt cc	63	266
TNFrI	att tgc tgt acc aag tgc cacc aaa gga acc	gtc cat ttc cca caa aca atg gag tag acg	65	587
TNFrII	caa gac ctc gaa cac cgt gtg tg	tat ccg tgg atg aag tcg tgt tgg aga acg	72	200
GAPDH	gtc aac gga ttt ggc cgt att gg	aaa gtt gtc atg gat gac ctt ggc c		

Table 3 - Frequencies for main effect and interaction (day 0/day 70) variables by specific amplimer found in the synovial membrane samples. Meaning, the number of samples with positive amplimers for the specific primer sequences.

Variable	MMP 1	MMP 3	MMP 13	IL-1Ra	vIL-1Ra	IL-1α	IL-1β	TNFα	TNFrI	TNFrII
Day 0	1	15	0	6	0	0	1	2	0	0
Day 70	0	27	0	25	1	13	24	20	0	0
No Chip	1	20	0	15	0	7	13	12	0	0
Chip	0	22	0	16	1	6	12	10	0	0
Placebo	0	0	0	15	0	5	12	10	0	0
Treated	1	24	0	16	1	8	13	12	0	0
No Chip Placebo	0/0	3/6	0/0	2/6	0/0	0/2	0/6	0/5	0/0	0/0
Chip Placebo	0/0	4/5	0/0	0/7	0/0	0/3	1/5	0/5	0/0	0/0
No Chip Treated	1/0	3/8	0/0	1/6	0/0	0/5	0/7	1/6	0/0	0/0
Chip Treated	0/0	5/8	0/0	3/6	0/1	0/3	0/6	1/4	0/0	0/0

Table 4 - Frequencies for main effect and interaction (day 0/day 70) variables by specific amplimer found in the articular cartilage from any location sampled within the joint.

Variable	MMP 1	MMP 3	MMP 13	IL-1Ra	vIL-1Ra	IL-1α	IL-1β	TNFα	TNFrI	TNFrII
Day 0	0	0	0	0	0	0	0	0	0	0
Day 70	0	18	2	7	1	4	10	22	23	10
No Chip	0	10	1	4	1	3	6	12	10	5
Chip	0	8	1	3	0	1	4	10	13	5
Placebo	0	8	1	2	0	1	5	7	10	3
Treated	0	10	1	5	1	3	5	15	13	7
No Chip Placebo	0/0	0/5	0/1	0/1	0/0	0/1	0/3	0/5	0/5	0/1
Chip Placebo	0/0	0/3	0/0	0/1	0/0	0/0	0/2	0/2	0/5	0/2
No Chip Treated	0/0	0/5	0/0	0/3	0/1	0/2	0/3	0/7	0/5	0/4
Chip Treated	0/0	0/5	0/1	0/2	0/0	0/1	0/2	1/8	0/8	0/3

5 Figures

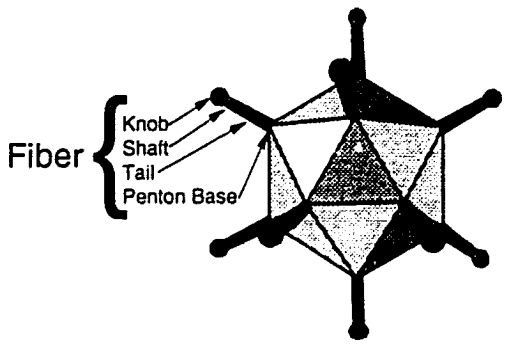

Figure 1 - The capsid structure has protruding from each of the 12 vertices a fiber protein made up of a tail, rod-like shaft and a terminal globular structure called the knob. (Adapted from Hitt et al. 1998)[46]

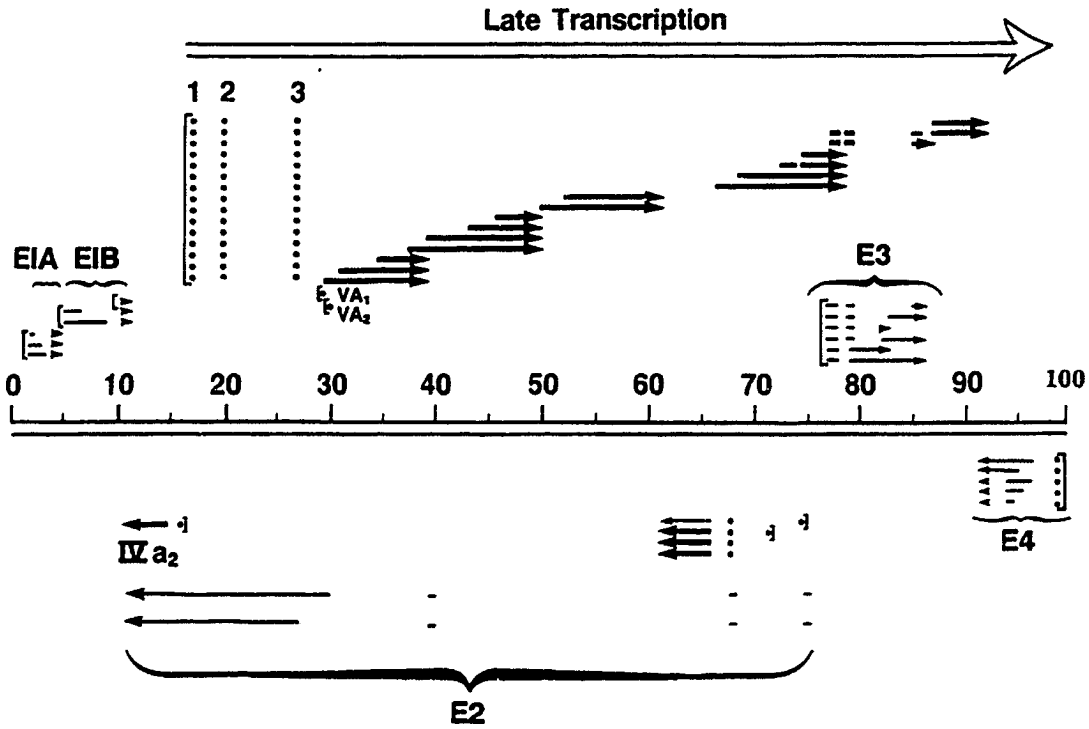

Figure 2 – Transcription map of adenovirus 5. Early adenoviral transcripts are shown as *light* arrows and labeled E1, E2, E3 or E4. The open arrow shows the primary late transcript, and the resulting processed late mRNAs are shown as *bold arrows*. The directions of the arrows indicate direction of transcription. 1, 2, and 3 indicates the tripartite leader of the late mRNAs. The genome length is given in map units with each map unit corresponding to 360 bp. (Adapted from Hitt et al. 1998)[46]

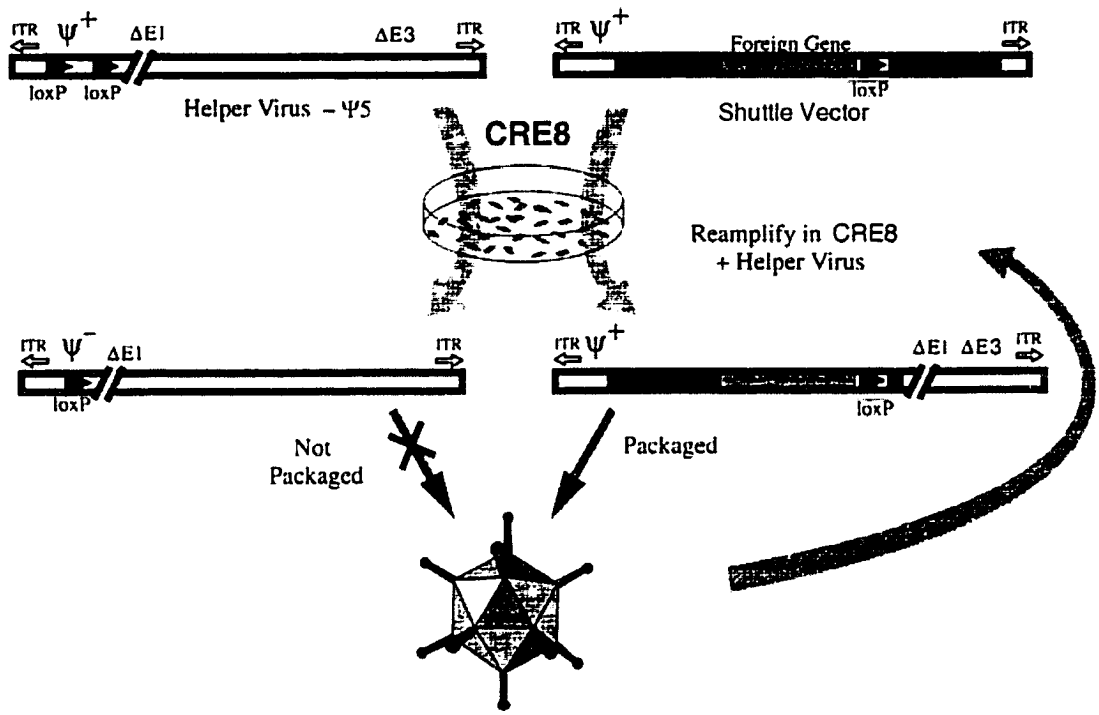

Figure 3 - An example of a Cre-*loxP* system for the generation of helper dependent adenoviral vector. ψ5 has a packaging signal (ψ^+) flanked by *loxP* sites such that upon transduction of CRE8 cells the packaging signal is excised rendering the ψ5 genome unpackageable. ψ5 is still able to function in the CRE8 cells and provide in *trans* all of the functions required for the replication and packaging of the shuttle vector. The titer of the recombinant vector is increased by serial passage through ψ5 transduced CRE8 cells. ψ5 can still be propagated using 293 cells that are Cre-recombinase negative. (Adapted from Hitt et al. 1998)[46]

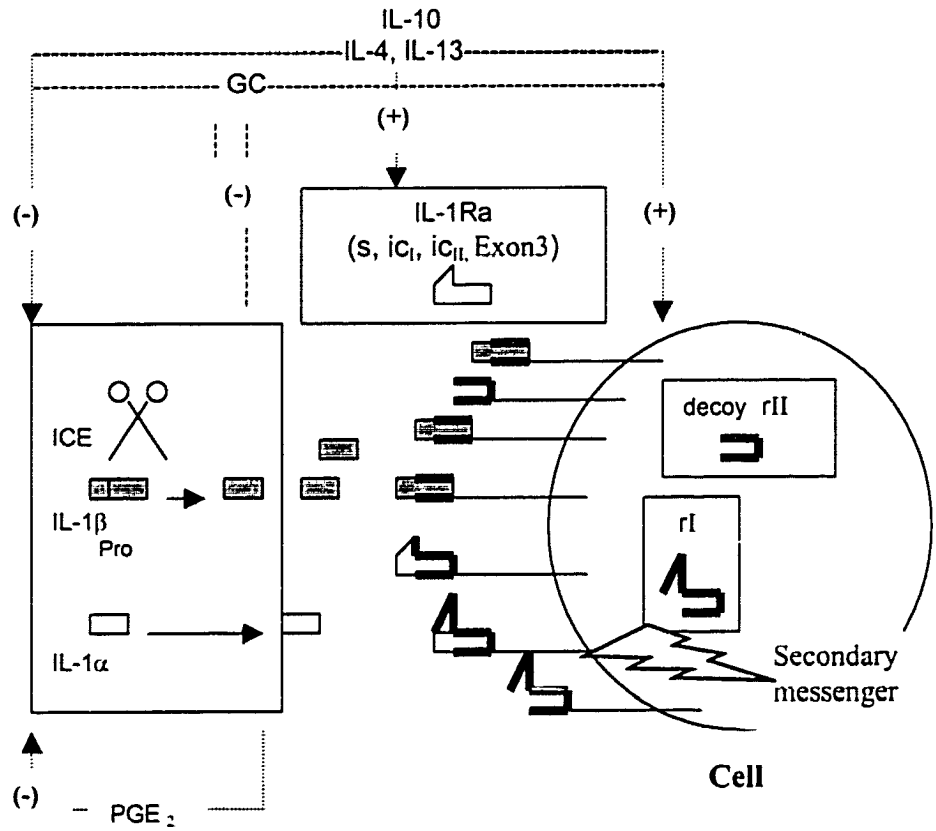

Figure 4 – A schematic representation of the "IL-1 system". The figure depicts regulatory pathways and their interaction with some of the IL-1 molecules.

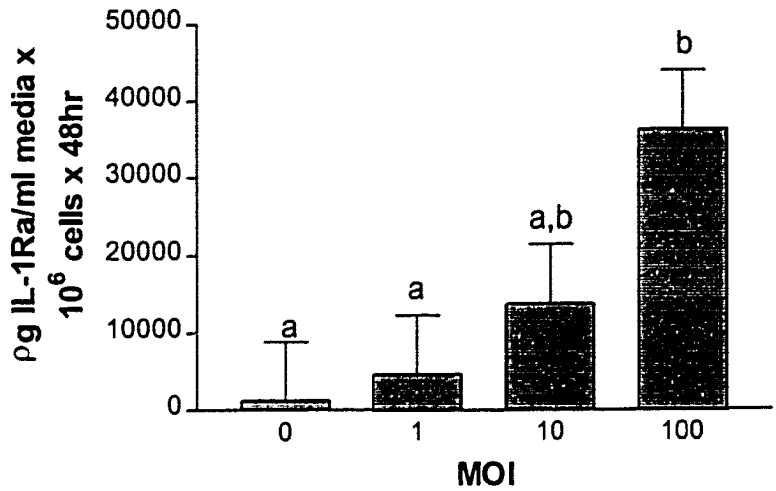

Figure 5 – The concentration of IL-1Ra measured in the media 48 hours post transduction of equine synoviocytes with various MOI concentration of Ad-EqIL-1Ra vector. The IL-1Ra concentrations are normalized to 1 x 10^6 cells, and different letters represent a statistical difference between groups using a P – value < 0.05.

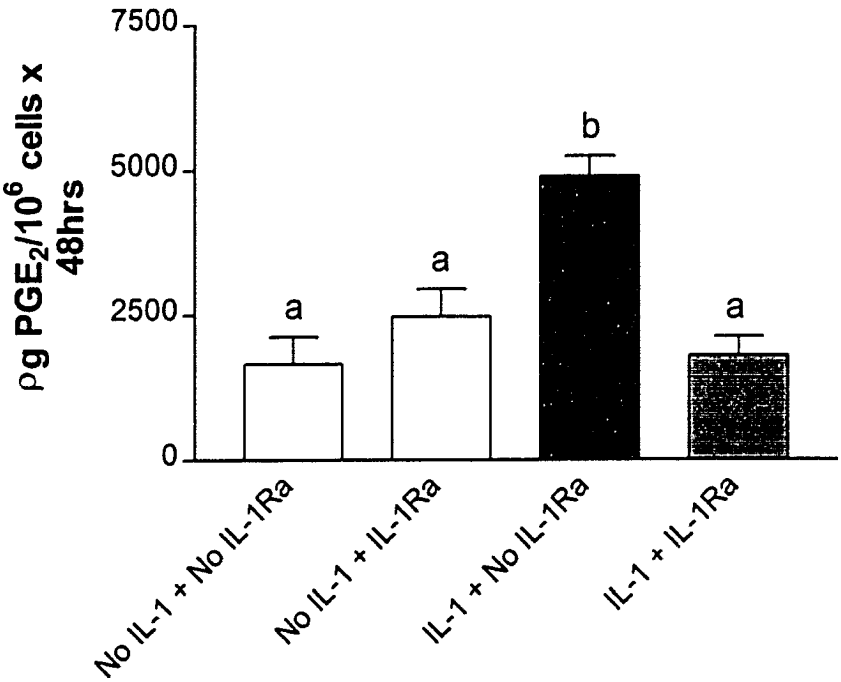

Figure 6 – The concentration of PGE_2 measured in the media by treatment group. The No IL-1 + No IL-1Ra treatment group were cells not exposed to IL-1α and non-transduced incubated for 48 hours. The No IL-1 + IL-1Ra treatment group were cells not exposed to IL-1α but transduced with Ad-EqIL-1Ra. The IL-1 + No IL-1Ra treatment group were exposed to IL-1α for 48 hours but non-transduced. The IL-1 + IL-1Ra treatment group were cells exposed to IL-1α for 48 hours and transduced with Ad-EqIL-1Ra (10 MOI). Different letters indicate a statistical difference between groups (P-value < 0.05).

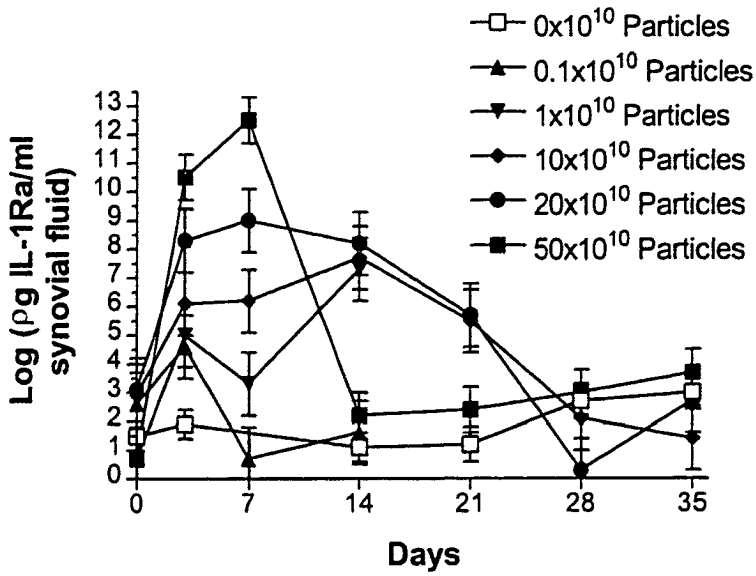

Figure 7 – The log of the IL-1Ra concentration measured in synovial fluid post transduction with various doses of Ad-EqIL-1Ra vector or placebo treatment (0 x 10^{10} particles).

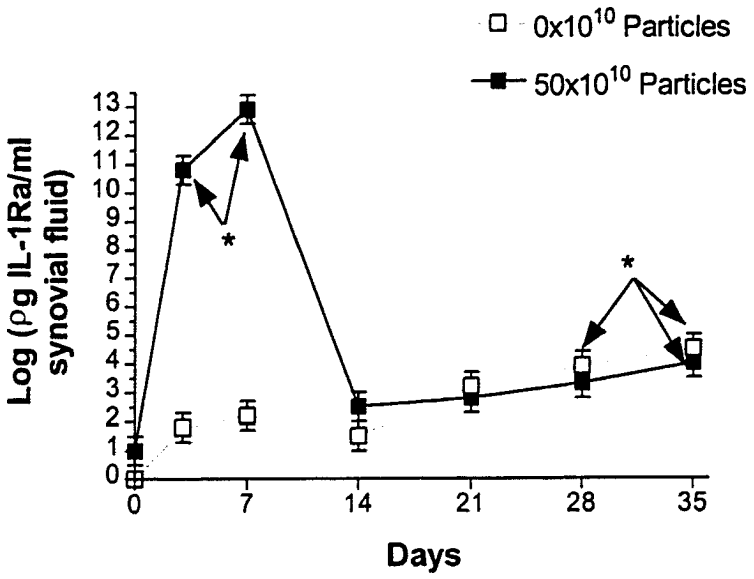

Figure 8 – The concentration of IL-1Ra measured in synovial fluid post transduction with either 0 (placebo) or 50 x 10^{10} particles/joint of the Ad-EqIL-1Ra vector. Asterisks (*) denotes a statistical difference (P – value < 0.05) in the data point compared to placebo treatment at days 0 – 14.

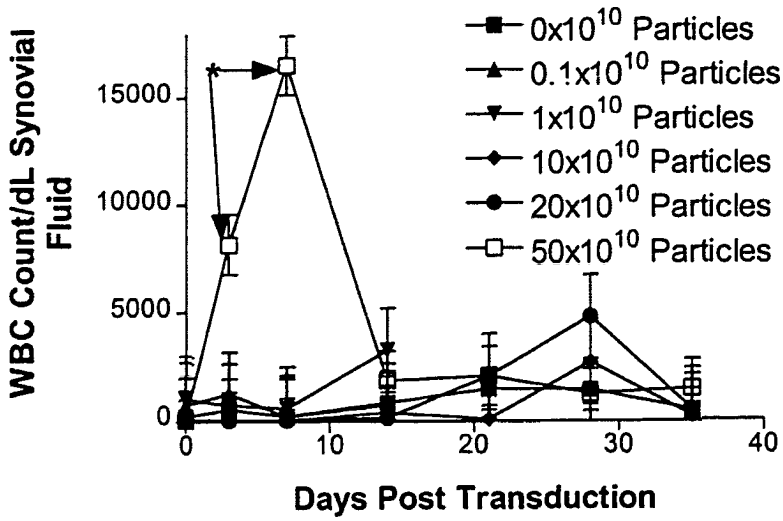

Figure 9 – The WBC counts measured in synovial fluid post transduction with various doses of Ad-EqIL-1Ra vector or placebo treatment (0 x 10^{10} particles). Asterisks (*) denotes a statistical difference (P – value < 0.05) in the data point compared to placebo treatment at that time period.

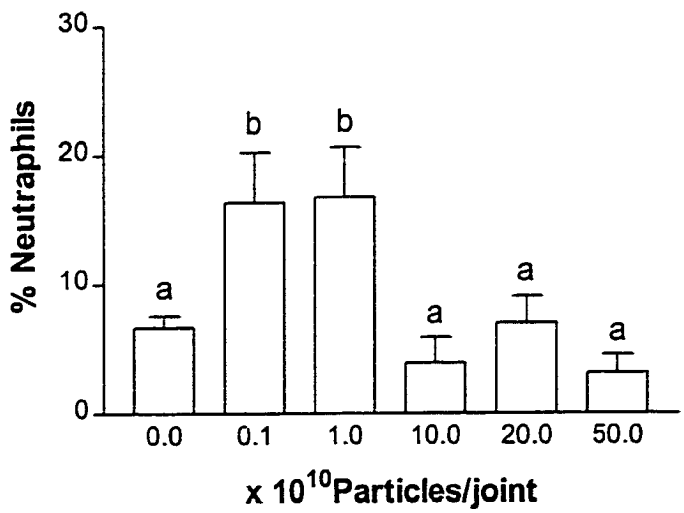

Figure 10 – The percent neutrophils comprising the WBC differential count from synovial fluid plotted by various doses of Ad-EqIL-1Ra vector or placebo treatment (0 x 10^{10} particles). Different letters represent a statistical difference between groups (P – value < 0.05).

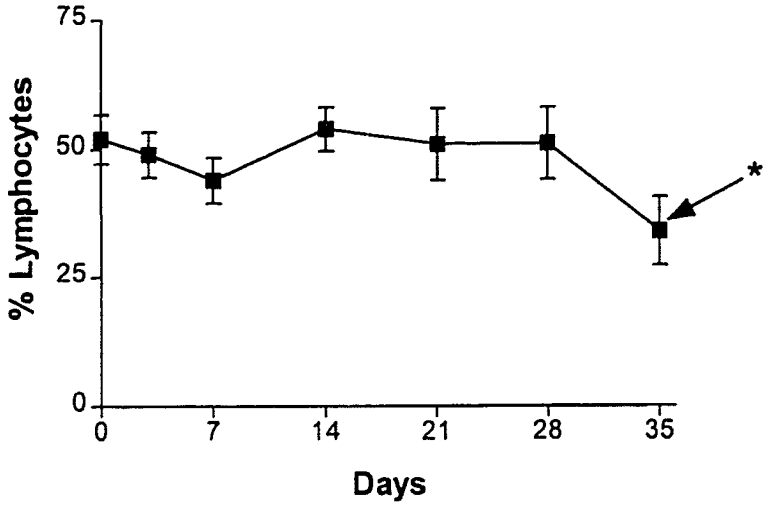

Figure 11 – The percent lymphocytes comprising the WBC differential count from synovial fluid samples plotted by days post transduction (averaged over all concentrations). Asterisks (*) denotes a statistical difference (P – value < 0.05) in the data point compared to levels at all other days except day 7 post transduction.

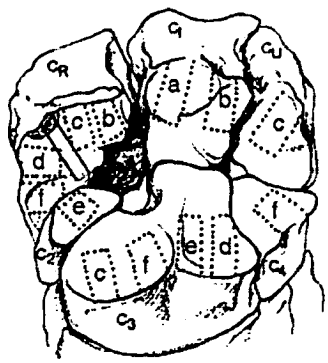

Figure 12 – Dorsal view of the equine intercarpal joint depicting areas of specific tissue sampling and the osteochondral fragment. A = area from which articular cartilage was harvested for estimation of PG synthesis. B = Areas from which articular cartilage was harvested for analysis of GAG content. C = areas from which articular cartilage was harvested for mRNA analysis. D = areas from which articular cartilage was harvested for future immunohistochemistry analysis. E = areas from which articular cartilage was harvested for future *in situ* hybridization analysis. F = areas from which articular cartilage was harvested for histopathology. The filled in area in the radiocarpal bone (C_R) represents the osteochondral fragment, the solid lines running through this region represent the section of bone harvested for routine histopathology. C_I = Intermediate carpal bone, C_U = Ulnar carpal bone, C_2 = Second carpal bone, C_3 = Third carpal bone, and C_4 = Fourth carpal bone.

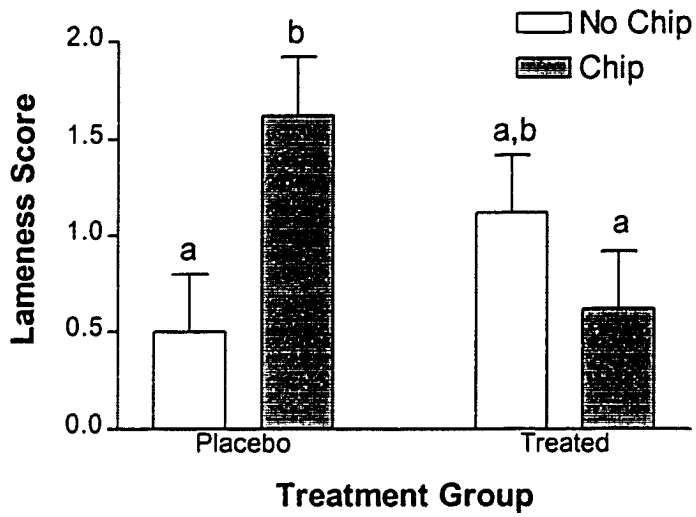

Figure 13 – Day 70 lameness scores plotted by treatment group. Different letters indicate a statistical difference (P – value <0.05) between bars.

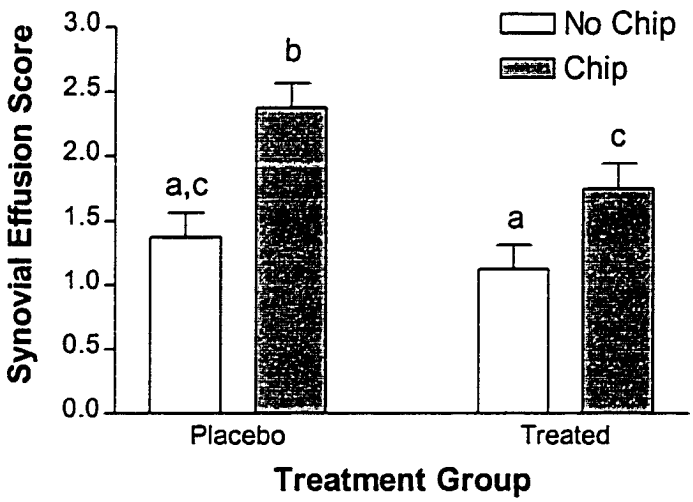

Figure 14 – Day 70 post-chip creation synovial effusion scores plotted by treatment group. Different letters indicate a statistical difference (P – value <0.05) between bars.

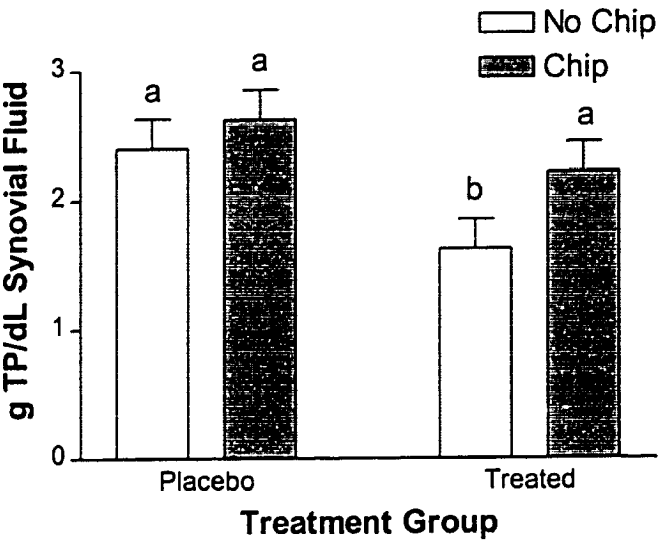

Figure 15 – Synovial fluid total protein plotted versus treatment group. Different letters indicate a statistical difference (P – value <0.05) between bars.

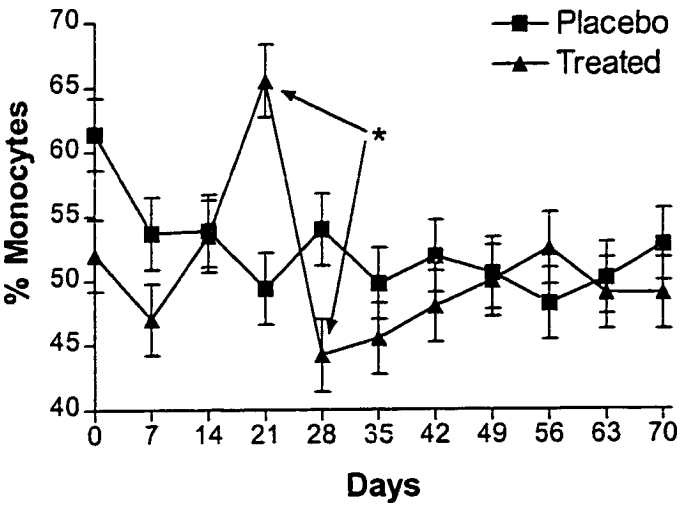

Figure 16 - The percentage of monocytes in the synovial fluid plotted for the average of joints from Placebo and Treated horse by days post-chip creation. An asterisk (*) denotes a statistical difference (P – value < 0.05) in the data point compared to placebo treatment at that time period.

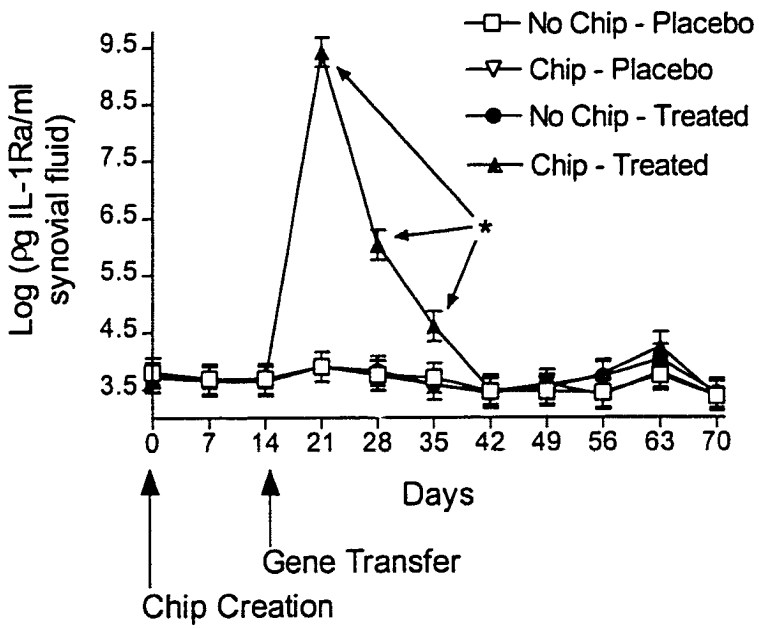

Figure 17 – Log of the IL-1Ra concentration measured in synovial fluid plotted by Days post chip creation. An asterisk (*) denotes a statistical difference (P – value < 0.05) in the data point compared to all other data points at that time period.

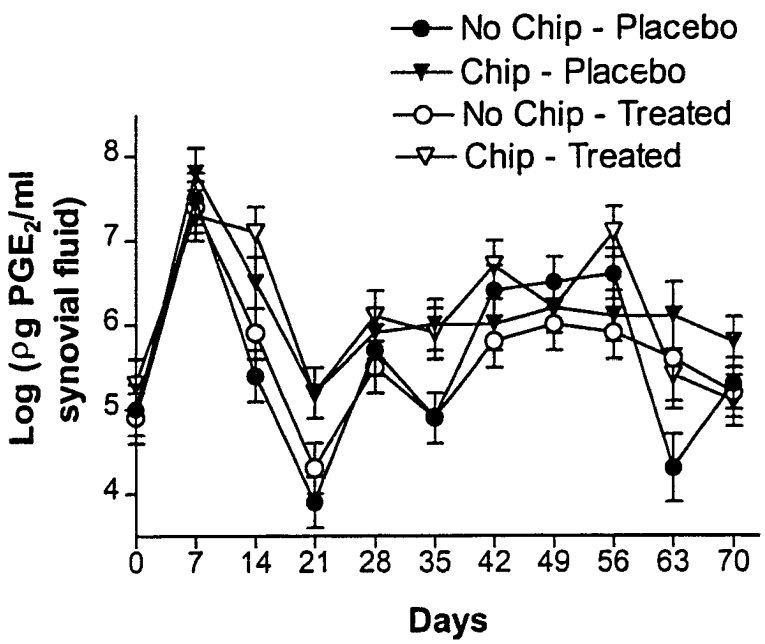

Figure 18 – Log of the PGE_2 concentration measured in synovial fluid plotted by days post chip creation. A significant increase in synovial fluid PGE_2 levels was demonstrated in all joints 7 days post-operatively, and on average in Chipped compared to No Chipped joints over all time periods. Treatment had no significant effect on synovial fluid PGE_2 levels.

Figure 19 – Photographs of the 3rd carpal bones from chipped joints of both Placebo (A) and Ad-EqIL-1Ra (B) treated horses. Note more extensive full-thickness articular cartilage erosions in the placebo treated joint especially in areas of the 3rd carpal bone not adjacent to the chip. Photos were taken after aseptic harvest of cartilage from the intermediate carpal bone.

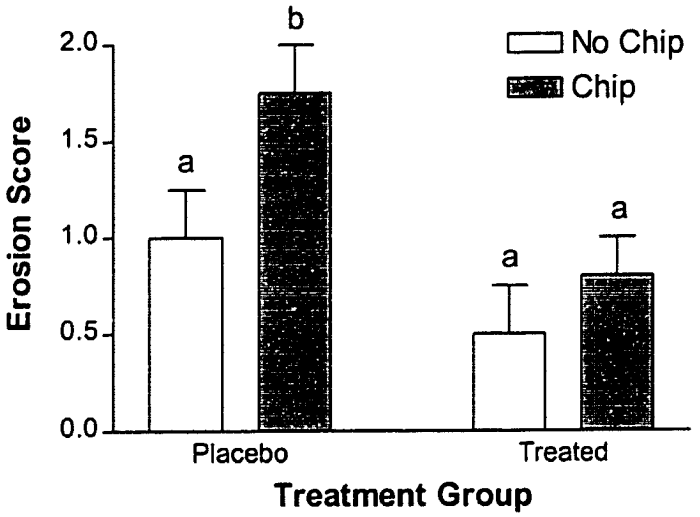

Figure 20 - Erosion scores from the 3rd carpal bone plotted by treatment group. Different letters associated with bars indicate a statistical difference (P – value <0.05) between bars.

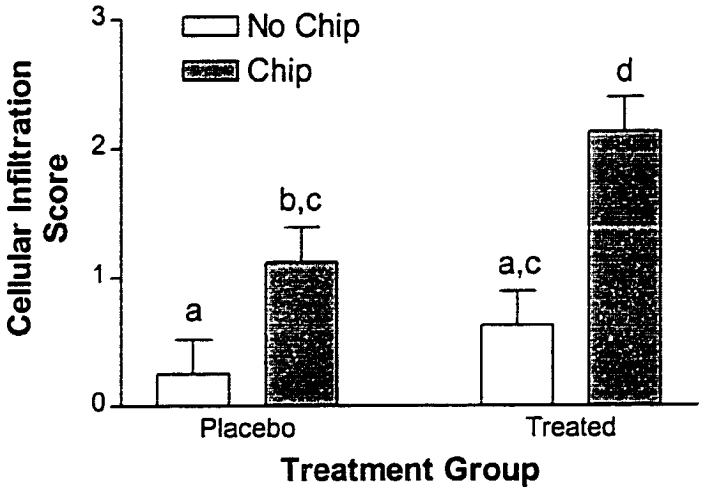

Figure 21 – The cellular infiltration scores from synovial membrane plotted by treatment group. Different letters indicate a statistical difference (P – value <0.05) between bars.

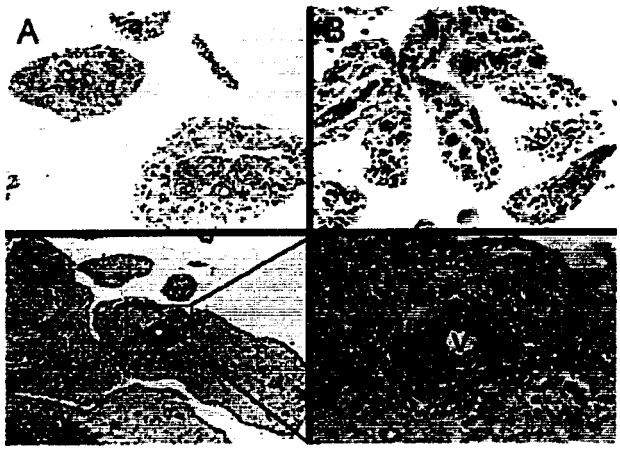

Figure 22 – Photomicrograph from 5μm sections of synovial membrane stained with H & E. Plate (A) is a representative area of synovial membrane with slight-mild increased vascularity harvested from a non-chipped joint of an Ad-EqIL-1Ra treated horse. Plate (B) is a representative area of synovial membrane with mild-moderated increased vascularity harvested from a chipped joint of a placebo treated horse. Plate (C) is a representative area showing one of the most severe examples of perivascular lymphocytic infiltration (L), neutrophils (N) and macrophages (M) can also be seen in the 40X blow-up (D) the tissue is from a chipped joint treated with Ad-EqIL-1Ra. Plates are at 4X unless otherwise noted.

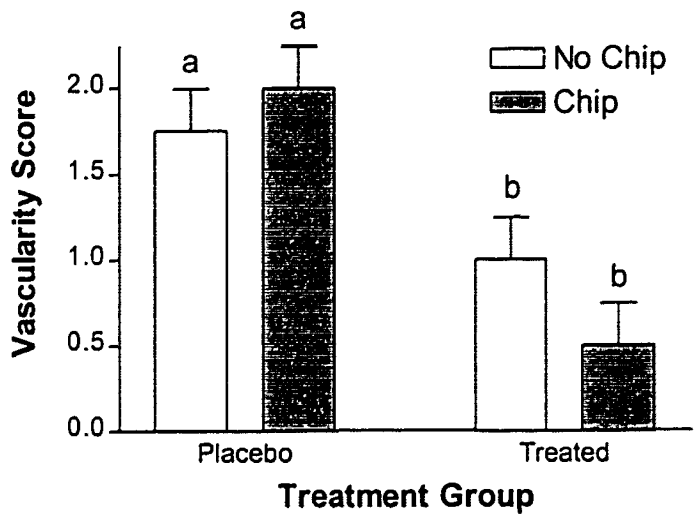

Figure 23 – Synovial membrane vascularity scores plotted by treatment group. Different letters associated with bars indicate a statistical difference (P – value <0.05) between bars.

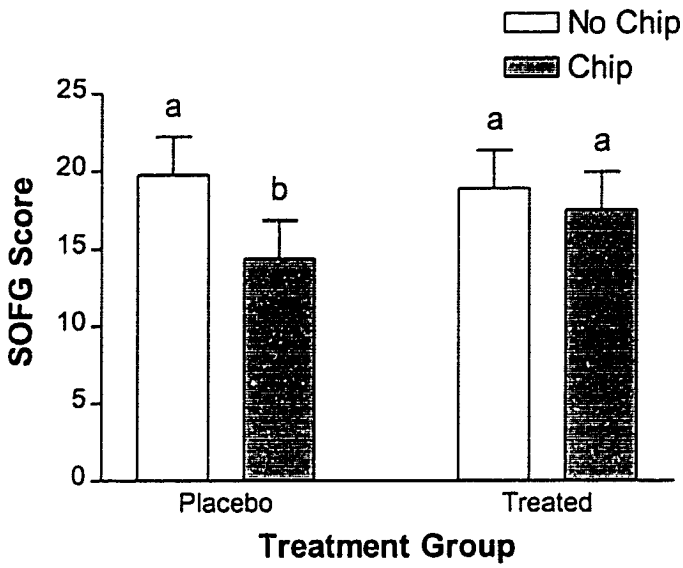

Figure 24 – Total score for SOFG staining from all measured areas within the joint and for all staining patterns plotted by treatment group. Different letters associated with bars indicate a statistical difference (P – value <0.05) between bars.

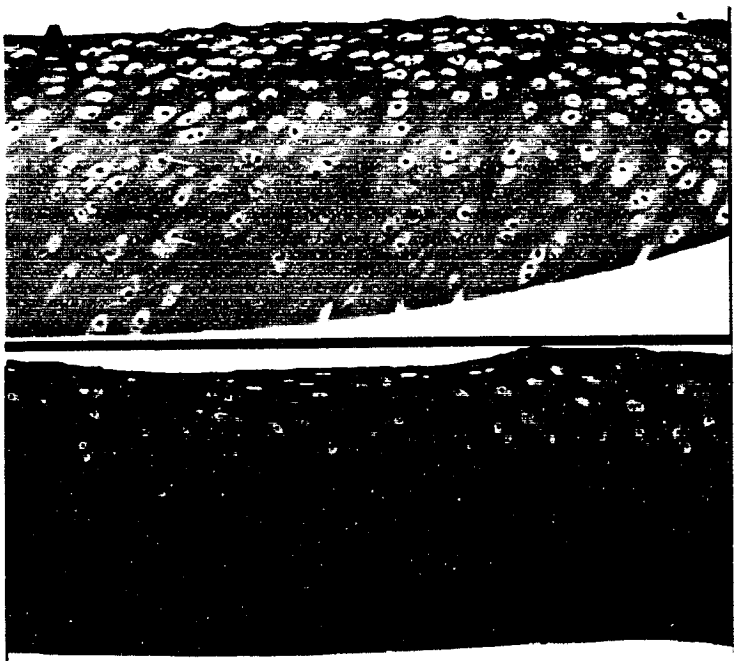

Figure 25 - Photomicrograph from 5μm sections of articular cartilage stained with SOFG. Plate (A) is a representative area of cartilage showing no to slight stain uptake patterns in all areas. The sample was harvested from a chipped joint of a placebo treated horse. Plate (B) is a representative area showing moderate stain uptake patterns in all areas The tissue was harvested from a chipped joint of an Ad-EqIL-1Ra treated horse.

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7 Endotes

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[i] Quantikine™ Human IL-1ra immunoassay, R & D Systems, Minneapolis, MN, USA.

[ii] TiterZyme® PGE_2 enzyme immunoassay kit, PerSpetive Biosystems, Inc., Framingham, MA, USA.

[iii] Clinical pathology services, Veterinary Teaching Hospital, Colorado State University, Fort Collins, CO, USA.

[iv] Life Technologies, Gaithersburg, MD, USA.

[v] Brinkmann Instruments, Inc., Westbury, NY, USA.

[vi] Promega, Madison, WI, USA.

[vii] Courtesy of Dr Dean Richardson, University of Pennsylvania, PA.

[viii] Courtesy of Dr John Caron, Michigan State University, MI.