AAV-mediated gene transfer to synovium: enhancing effects of inflammatory cytokines and proteasome inhibitors

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AAV-mediated gene transfer to synovium: enhancing effects of inflammatory cytokines and proteasome inhibitors

Russell S. Traister, PhD

University of Pittsburgh, 2006

Rheumatoid arthritis (RA) is an autoimmune disease affecting nearly 1% of the population. The joints of RA patients become inflamed and a pannus of synovial tissue invades cartilage and destroys bone. Current treatments, though moderately successful, are often administered systemically and are associated with significant side effects. Local treatment of arthritis by intra-articular injection of gene transfer vectors has the potential to overcome some of the obstacles of other treatment regimens. In particular, adeno-associated virus (AAV) is attractive as a gene transfer vector because it is non-pathogenic in humans, has low immunogenicity and broad tissue tropism, and provides for long-term transgene expression. The primary target of gene transfer in the joint is the fibroblast-like synoviocytes (FLS), which make up part of the pannus of tissue responsible for cartilage and bone destruction. However, FLS are only minimally transduced by AAV and provide only low levels of transgene expression. For local treatment with AAV vectors to be successful, improvement in the transduction of FLS is necessary. Several agents have been shown to increase AAV transduction in other cell types. The specific effect of inflammation and proteasome inhibition on AAV-mediated transgene expression in FLS has not been examined. Here we show that both inflammatory cytokines and the proteasome inhibitor zLLL can increase transgene expression in FLS dramatically. Remarkably, both agents are also able to regulate transgene expression. The cytokines appear to act in part by promoting uncoating of the virus, while proteasome inhibition works by both increasing the nuclear

trafficking of the virus and by increasing transcription of the viral DNA. In addtion, we screened 3 serotypes of AAV (2, 2.5, and 5) for their ability to transduce FLS from 4 different species: human, mouse, horse, and rabbit. AAV2 provided the highest transgene expression in human and horse FLS, while AAV2.5 was better suited for mouse and rabbit FLS. These results have implications in the future use of AAV vectors in the treatment of RA.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

Scientific research is, to me, one of the most thought provoking and engaging disciplines. I have been interested in all things scientific from a young age. In high school, biology was always one of my favorite subjects. In my spare time, books like Jurassic Park heightened my interest in all things scientific. I have also always had an interest in puzzles, logic games, and strategy games. I feel that science is like a giant puzzle, with lots of pieces that must be put together in an organized and well thought out manner. With this in mind, I hope to use what I've learned in the production of this thesis as the beginning to a long and successful research career.

I could not have completed this thesis work without the support of a lot of people. First and foremost is my advisor and mentor, Dr. Raphael Hirsch. His ability to blend a career in both science and medicine is a model that I hope to follow. Dr. Hirsch also treats his students and everyone in his lab as equals, with everyone's input considered welcomed and important. This inspires independent thinking and encourages cooperation between lab members. He also was always available for advice at a moments notice. In addition, the input of the fellow members of the lab, including Dave, Tony, Takako, Soam, Margalit, and Suzanne has been invaluable. Thanks also to my committee members Dr. Penelope Morel, Dr. Xiao Xiao, Dr. Paul Robbins, and Dr. Jay Kolls for their advice and guidance during my time in the lab. Thanks also to my roommates Lou, Helen, and Casey for their advice and support. Being able to seek advice from people who have been through all of this ahead of me made my trek that much easier. And lastly, thanks to my family for all of their support. It has been a long road but I promise one of these days I will graduate!

1. Introduction

1.1. Overview of Rheumatoid Arthritis

1.1.1. Epidemiology of Rheumatoid Arthritis

Rheumatoid arthritis (RA) is the most common inflammatory disorder, affecting approximately 0.5-1% of the North American adult population. It causes significant pathology and functional impairment in affected individuals, leading to an increased mortality, with survival reduced by an average of 3-10 years. RA it is a multi-factorial disease whose main risk factors include genetic susceptibility, sex and age, smoking, infectious agents, hormones, diet, and socioeconomic and ethnic factors (1). Those afflicted by RA report a decrease in quality of life measures such as persistent pain, functional disability, fatigue, depression, and an inability to perform daily tasks (2). RA is also a significant burden on the health care system, averaging between $2800-$28,500 per patient per year for direct and indirect costs (3).

1.1.2. Pathogenesis of Rheumatoid Arthritis

Although the pathogenesis of RA is not completely understood, specific HLA-DR genes, autoantibody and immune complex production, T cell antigen specific responses, networks of cytokine production, and a hyperplastic, aggressive synovium have all been shown to play a role. RA primarily affects diarthrodial joints of the hands and feet. The joint is normally lined by a thin cell layer (1-3 cells thick) of both type I (macrophage-like, MLS) and type II (fibroblast-like, FLS) synoviocytes (4).

FLS make up approximately 2/3 of the synoviocytes. They have a spindle-like shape and an extensive endoplasmic reticulum. They originally develop between foci of cartilage in the embryonic limb bud. FLS secrete molecules like hyaluronan and lubricin, which act to lubricate

the joint and contribute to the viscosity of the synovial fluid. The synoviocytes are also responsible for maintaining synovial fluid volume, a breakdown in which can lead to increase mechanical stress on the joints. They also play a key role in controlling inflammatory responses, with effects on complement fixation and migration of immune cells within the synovium and into the joint space. FLS also help maintain the structure of the joint capsule through secretion of connective tissue components such as fibronectin, proteoglycans, and laminins (5).

In RA, the joint becomes inflamed and the synovium becomes hyperplastic, creating a pannus of synovial tissue comprised of CD4+ T cells, B cells, mast cells, dendritic cells, macrophages, and synoviocytes that invade and destroy nearby cartilage and bone. The synovial cell layer expands to a depth of 15 or more cells. FLS secrete growth factors that contribute to their own proliferation. Mutations in gene that regulate proliferation, such as *p53*, *Erk*, and *Z-225* may also occur. There are also decreases in apoptosis rates of FLS, associated with decreased Fas-mediated apoptosis, and overexpression of bcl-2, among others. FLS also play a role in the inflammation observed in RA. FLS secrete numerous pro-inflammatory cytokines and pro-angiogenic factors. FLS can also interact directly with T cells, inducing T cell activation (5). Neutrophils accumulate in the synovial fluid and also contribute to the degradative processes of RA (4, 6). Macrophage and fibroblast-like synoviocytes secrete cytokines such as TNFα, IL-1β, and IL-6, among others, all of which perpetuate cartilage and bone destruction (7, 8). These cytokines are thought to act primarily through mitogen activated protein kinase (MAPK) and nuclear factor κB (NFκB) signaling pathways to activate transcription factors that turn on genes for chemokines and cell adhesion molecules, along with extracellular matrix degrading enzymes like matrix metalloproteinases (MMPs). Chemokines and chemokine receptors have also been linked to RA (7, 9, 10).

1.1.3. Treatment for Rheumatoid Arthritis

Initial treatment strategies for RA include drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids. While providing pain relief and decreased joint swelling, these drugs are unable to stop progression of RA (11). This led to the use of small molecules such as methotrexate and penicillamine that were demonstrated to slow disease progression. For many decades these disease-modifying agents of rheumatic disease (DMARDs) were the best treatment option for RA. In recent years, a further understanding of the disease process has led to the development of biologic DMARDs primarily aimed at neutralizing the effects of pro-inflammatory cytokines. The most successful agents in this class are anti-TNFα molecules and IL-1β blocking agents. Based on improvement criteria set forth by the American College of Rheumatology (ACR), these drugs are more effective than treatment with methotrexate alone. Even so, less than half of the patients show improvement of at least 50% in their ACR scores. In addition, the half-life of these drugs is relatively short and they require frequent systemic administration in order to be effective (12). Gene transfer strategies have the potential to overcome some of these limitations, potentially leading to increased efficacy and decreased frequency of administration.

1.2. Gene Delivery Strategies

1.2.1. Systemic versus local administration strategies

Although the majority of inflammation in RA is locally in the joints, there are systemic components to the disease. Therefore, when developing treatment strategies, one must consider whether the therapy should be delivered locally or systemically. Local administration is attractive because it has less potential for side effects and the treatment is delivered directly to the joint, the main site of inflammation. However, utilizing this strategy, systemic features of the

disease would be left untreated. Interestingly, several researchers have observed a "contralateral effect" in animal models of local gene delivery where the delivered transgene is protective not only to the injected joint, but also to distal, untreated joints (13-15). This effect appears to be independent of trafficking of modified immune cells to distal joints, demonstrated by the ability of *ex vivo* modified fibroblasts alone to confer the contralateral effect (16). The contralateral effect is also independent of systemic circulating levels of transgene and non-specific immunosuppression and instead was found to depend on a complex antigen specific mechanism (17). Systemic delivery, typically by intravenous (i.v.) administration, would be expected to have a broader therapeutic effect, but is also associated with an increase in side effects and toxicity.

1.2.2. *In vivo* versus *ex vivo* strategies

Another consideration for gene transfer is whether to employ *in vivo* or *ex vivo* delivery strategies. *In vivo* strategies have the advantage of being relatively easy and inexpensive. In addition, many more studies have been performed in animal models looking at *in vivo* gene delivery. *Ex vivo* strategies, although expensive and time consuming, have the advantage of being able to treat and select very specific cells, avoiding the possibility that the gene transfer vector could genetically modify cells that are desired to remain undisturbed from their natural state. A phase I clinical trial has been performed using a retroviral vector to deliver IL-1 receptor antagonist (IL-1ra) to cultured autologous synovial fibroblasts. The cells were then injected into RA patient joints. After a scheduled arthroplasty one week later, significant expression of the transgene was observed in the injected joints. No adverse events were reported (18). This trial was initiated following previous experiments where antagonists of both IL-1 and

TNF were delivered to autologous cultured rabbit fibroblasts *ex vivo* and injected into arthritic rabbit knee joints, with significant therapeutic benefit (16, 19).

Most *ex vivo* studies use fibroblast-like synoviocytes (FLS) as the target cell. These cells are targeted specifically because they are thought to be directly responsible for cartilage destruction and drive and perpetuate the inflammatory response and autoimmunity (5). The disadvantages of using this cell type are that FLS have a low proliferation rate, lack highly specific surface markers, and are a non-homogenous population. Alternatively, future studies may examine other strategies, including gene delivery to T cells, dendritic cells, muscle cells, or mesenchymal stem cells (20).

1.3. Gene transfer vectors

Gene transfer vectors can be broadly categorized into two groups: viral and non-viral vectors. In general, viral vectors tend to provide for longer-term gene expression but often come with additional safety concerns, including fears of generating replication competent virus during vector production, random insertion of the transgene into the genome following treatment, or development of a harmful immune response. Viral vectors are by far the most widely used vectors for delivering transgenes in arthritic animal models (21). There are several different viral vectors that have been examined for use in gene transfer for arthritis, including adenovirus, retrovirus, adeno-associated virus (AAV), and lentivirus, each with their respective advantages and disadvantages. Non-viral vectors are often easier to make and come with less concerns about contamination. The most common non-viral vector used in arthritis studies is plasmid DNA.

1.3.1. Non-viral vectors

1.3.1.1. Plasmid DNA

Plasmid DNA can be delivered by lipsomes, gene gun, or direct injection of the plasmid. The use of plasmid DNA tends to be less toxic and less immunogenic than use of viral vectors and is also easy and relatively inexpensive to produce. However, plasmid DNA often leads to low transfection efficiency and only short-term expression of the transgene, often for only 1-2 weeks and typically peaking even earlier (22-24). These limitations make it unlikely that local delivery of plasmid DNA in the joint will be successful.

The most success with the use of plasmid DNA in gene transfer for arthritis has been garnered through delivery of transgenes to skeletal muscle. Electrotransfer of variants of soluble TNF-α receptor I variants to the tibial-cranial muscle at the onset of collagen-induced arthritis (CIA) led to a decrease in the clinical and histological signs of disease for up to 5 weeks after treatment (25). Similarly, plasmids encoding cDNA for other anti-inflammatory molecules such as IL-1ra and a soluble TNF-Fc fusion protein have been demonstrated to improve both macroscopic and microscopic scores of CIA when delivered intra-muscularly (26, 27). A plasmid encoding TGF-β delivered to skeletal muscle delayed progression of streptococcal wall induced arthritis when administered at the peak of the acute phase and virtually eliminated subsequent inflammation and arthritis when given at the beginning of the chronic phase of the disease (28). Intra-muscular injections of plasmids encoding immuno-modulatory molecules such as IL-4, IL-10, viral IL-10, and soluble complement receptor type I have also demonstrated beneficial effects in small animal models of arthritis (29-32).

Intramuscular delivery of a plasmid encoding TIMP-4, an inhibitor of matrix metalloproteinases, completely abolished the development of arthritis in a rat adjuvant-induced arthritis model (33). Intravenous delivery of a plasmid encoding the heparin-binding domain of

fibronectin inhibited leukocyte recruitment and decreased inflammation in CIA (34). Intradermal injection of plasmid encoding IL-10 and intra-peritoneal injection of plasmid IL-10/liposome complexes have also been demonstrated to delay onset and progression of CIA (35, 36). The liposome delivered DNA was able to maintain expression for only 10 days after injection, significantly less than the intramuscular studies mentioned above.

1.3.1.2. Other non-viral vectors

Other non-viral gene delivery systems that have potential in the treatment of RA are the artificial chromosome expression (ACE) system and the sleeping beauty (SB) transposon system. The ACE system is attractive because it is non-integrating and can provide stable and long-term gene expression of one or multiple genes. A feasibility study was recently performed in a *Mycobacterium tuberculosis* rat arthritis model demonstrating that rat skin fibroblasts could be modified *ex vivo* to express a reporter gene from an artificial chromosome. These cells, when subsequently injected into rat joints, demonstrated engraftment into the synovial tissue microarchitechture and detectable transgene expression. The ACE system also had a lack of local inflammation at the injection site that is often associated with viral vector administration (37). The sleeping beauty transposon system melds the advantages of both viral and non-viral vectors, allowing for both integration into the genome and long term expression. No studies have yet been performed in arthritis models using the sleeping beauty transposon system, but success has been found in both cancer and hemophilia models, suggesting it might be have potential to successfully treat arthritis as well (38).

1.3.2. Viral vectors

1.3.2.1. Adenovirus

Adenovirus is a non-enveloped double-stranded DNA virus that can infect non-dividing cells and can be produced at high titers. Many gene therapy studies have been performed with this vector but it has several limitations that may prevent it from having success in the clinic. The high sero-prevalence of neutralizing antibodies to adenovirus may prevent successful administration or re-administration. Injected adenovirus also causes a significant inflammatory immune response, which is a safety concern. In addition, adenovirus vectors typically only allow for 1-3 weeks of transgene expression, which would severely limit its long-term efficacy. Some improvements to adenoviral vectors have recently been made in an effort to improve delivery of transgenes to the synovium. Fibroblast-like synoviocytes (FLS) lack the coxsackie-adenovirus receptor (CAR) and are not efficiently transduced by adenovirus. By modifying the fiber knobs on the virus, adenoviral transgene delivery to synoviocytes and synovium was improved dramatically (39, 40). Other recent improvements include the development of an adenoviral vector with an inflammation inducible promoter (41). This would allow expression of the transgene during active disease, but expression would turn off once inflammation was brought under control.

1.3.2.2. Retrovirus

Retroviruses, mostly derived from the Moloney murine leukemia virus, have a relatively simple genome and structure. They are enveloped viruses and contain two identical copies of their RNA genome. The key feature of the retroviral life cycle is the ability of the RNA genome to be reverse transcribed into double-stranded DNA, which can then randomly integrate into the genome. They have been mostly used in *ex vivo* studies and are desirable vectors for several reasons. They can provide for long-term stable expression and their integration into the genome makes it possible to permanently correct a genetic defect (42). For arthritis in particular, the

inflamed synovium appears to be more susceptible to uptake of the virus (19). The drawbacks to retroviral vectors are that they only infect non-dividing cells and are produced at low titers. The fact that these vectors integrate into the genome randomly is also a concern. In fact, in a recent clinical trial in France using a retrovirus to correct an X-linked SCID disorder, 3 out of 10 children developed leukemia after the vector inserted in or near a known oncogene. As a result, similar trials in the U.S. for this disorder have been halted until more information can be gathered (43, 44). Future improvements to these vectors, including the development of self-inactivating vectors, which contain no retroviral promoter or enhancer elements, and use of vectors from non-oncogenic retroviruses will hopefully make them safer for clinical use (42).

1.3.2.3. Lentivirus

Lentivirus vectors are derived from retroviral vectors but have the advantage of infecting non-dividing cells. The most commonly studied lentiviral vectors are derived from either human immunodeficiency virus (HIV) or feline immunodeficiency virus (FIV), although equine anemia infectious virus and visna virus have also been examined (38). The primary concern with HIV vectors is safety. In contrast, FIV is non-pathogenic in humans and does not cause serologic conversion (45). Using an FIV vector, TNF-α was transduced into primary human FLS with high efficiency. When injected into knees of SCID mice these cells induced cell proliferation and caused bone and joint destruction (46). A replication defective HIV vector encoding endostatin was injected into joints of TNF-α transgenic mice and was shown to decrease synovial blood vessel density and decrease the overall arthritis index (47). Similarly, intrarticular expression of angiostatin inhibited the progession of CIA in mice (48). A study examining a VSV-G pseudo-typed HIV vector, which has increased host range and stability,

demonstrated that a transgene could be efficiently delivered to the synovium of rat knee joints, with trangene expression lasting up to 6 weeks in immunocompromised animals (49).

1.3.2.4. Adeno-Associated virus

One of the most promising gene transfer vectors is adeno-associated virus (AAV). AAV is a small, non-enveloped single stranded DNA virus with broad tissue tropism. It belongs to the Parvoviridae family and has a 4.68kb genome. AAV normally requires adenovirus or herpesvirus to produce active infection. Several serotypes have been identified in primates, with AAV2 being the prototype for most gene transfer studies. AAV is an attractive vector for gene transfer studies for several reasons. It has been shown to deliver transgenes to a wide variety of tissues, has low immunogenicity, and mediates long-term gene expression (50). Recombinant, replication incompetent AAV vectors have been designed that lack the *Rep* gene, which is required for integration, so long term expression with these vectors is thought to be mediated by episomal viral DNA (51). In addition, AAV vectors have been designed that are able to package double stranded viral genomes, bypassing a rate-limiting step of viral transduction (second strand synthesis) and allowing rapid and highly efficient transduction both *in vitro* and *in vivo* (52).

Primary and recurrent arthritis were suppressed following a single injection of AAV encoding IL-1ra into knee joints of rats with LPS-induced arthritis. Surprisingly, disease regulated expression of the transgene was observed (53). AAV encoding soluble TNFα receptor type I decreased synovial cell hyperplasia and cartilage and bone destruction in human TNF-α transgenic mice injected intra-articularly with the virus (54). Intra-articular or peri-articular delivery of AAV encoding IL-4 in CIA mice was also shown to decrease paw swelling, protect from cartilage destruction and delay the onset of CIA (55, 56). A vIL-10 transgene delivered by AAV under control of a tetracycline inducible promoter decreased the incidence and severity of

CIA on a macroscopic, radiologic, and histologic level (57). More recently, angiostatin, an anti-angiogenic molecule, was demonstrated to efficiently decrease development of CIA in the treated joint when delivered by AAV (58).

AAV vectors are in clinical trials for the treatment of several diseases and preliminary results are promising (59). Targeted Genetics is currently conducting a phase I clinical trial (13G01; identifier NCT00126724) to assess the safety of using an AAV2 vector to deliver a soluble TNF receptor-Fc fusion gene in rheumatoid arthritis patients.

1.4. Candidate genes

After selecting a suitable vector for gene transfer studies, the next most important factor is the choice of the transgene that the vector will be used deliver. As mentioned above, RA is a multi-factorial disease. This provides a wide range of candidate genes with the potential to successfully treat RA. Several different strategies have been employed. Inhibitors of cytokines act to down-regulate inflammation. Immune deviation attempts to modulate the self-destructive immune response that is characteristic of autoimmune diseases like RA. Apoptosis-promoting transgenes help curb the hyper-proliferating and destructive pannus of tissue in the joint. Anti-angiogenic factors aim to prevent the neovascularizton of the proliferating pannus. Other strategies target matrix degradation enzymes or the NFκB transcription factor. Each approach is discussed in detail below.

1.4.1. Inhibitors of cytokines

1.4.1.1. Inhibitiors of IL-1β

Several gene transfer strategies have been aimed at neutralizing the effects of IL-1β. IL-1β communicates with many different cell types in the joint. Its action on these cells leads to emigration of blood cells to the synovium, increased cartilage destruction, and increased

production of other key chemokines and pro-inflammatory mediators by other cell types, including macrophages, B cells, and T cells (8). Neutralization of this key cytokine has proven beneficial in the treatment of RA. The use of gene transfer to block the effects of this cytokine is supported by several animal models.

Primary and recurrent arthritis were suppressed following a single injection of AAV encoding IL-1ra into knee joints of rats with LPS-induced arthritis (53). Adenoviral vectors encoding IL-1ra proved more effective at reducing cartilage matrix degradation and decreasing leukocyte infiltration into the joint space than vectors encoding a soluble type I TNF receptor-IgG fusion protein when injected into the knees of rabbits with antigen induced arthritis (14). Intramuscular injection of plasmid DNA encoding IL-1ra has also been shown to decrease paw swelling and arthritis incidence in a CIA mouse model. Reduced synovitis and cartilage erosion were also seen (27, 60). In a rat model of bacterial cell wall induced arthritis, rat synoviocytes were modified *ex vivo* using a retroviral vector to express IL-1ra. When injected into ankle joints prior to reactivation of arthritis, a decreased severity of arthritis and attenuated destruction of cartilage and bone was observed (61).

In a SCID mouse model, human RA FLS transduced with retrovirus encoding IL-1ra co-implanted with normal human cartilage was able prevent progressive cartilage degradation compared to controls (62). 3T3 mouse fibroblasts transfected with plasmid encoding IL-1ra were able to prevent onset of CIA and cartilage destruction when injected into knee joints of CIA mice (13). Similar effects on arthritis were seen in a rabbit model of antigen-induced arthritis in which a retroviral vector carrying the IL-1ra gene was used to tranduce rabbit fibroblasts *ex vivo*, with subsequent injection of the cells into the knee joint (16). More recently, the soluble form of interleukin-1 receptor accessory protein (sIL-1RAcP) was delivered to CIA mice either using an

adenoviral vector or by injection of plasmid transfected 3T3 cells. In both instances a profound prophylactic effect on the development of CIA was observed (63).

1.4.1.2. Inhibitors of TNF-α

TNFα is another pro-inflammatory cytokine that plays a key role in the pathogenesis of RA. Many of this cytokine's effects overlap those of IL-1β listed above. Currently, one of the most successful treatment strategies for RA, Enbrel (Immunex Corporation), is aimed at neutralizing TNFα. Treatment with Enbrel requires subcutaneous injections twice weekly. A gene tranfer vector encoding this molecule has the potential to only require only one or very infrequent administration, an obvious benefit over the existing regimen. After one injection at onset of CIA, intramuscular electro-transfer of plasmids encoding soluble TNF receptor I variants led to a decrease in clinical and histological signs of CIA (25). Expression lasted up to five weeks and was as least as efficient as repeated injections of the recombinant protein etanercept in controlling the disease. A similar study using a retroviral vector to deliver the trangene peri-articularly saw similar results and also observed a decrease in systemic levels of IgG2a antibodies to collagen type II (64). Electro-transfer of a plasmid encoding a soluble p75 TNF receptor:Fc fusion protein was also beneficial in a CIA model and was associated with a decrease in the levels of IL-1β and IL-12 in the paw (26). Another study using electrotransfer of a plasmid with a doxycycline-regulated promoter to control expression of a dimeric soluble TNF receptor II molecule saw a therapeutic effect on CIA only when doxycycline was administered (65). In TNF-α transgenic mice, intra-articular delivery of soluble TNF receptor I by adeno-associated virus led to a decrease in synovial cell hyperplasia and lead to a decrease in cartilage and bone destruction (54).

Splenocytes from arthritic DBA-1 mice can passively transfer collagen type II-induced arthritis when injected into SCID recipients. If these splenocytes were first modified *ex vivo* using retroviral vectors to express soluble p75 tumor necrosis factor receptor, the SCID recipients did not develop arthritis, bone erosion, or joint inflammation (66, 67). Delivery of a rat TNF receptor:Fc fusion protein in a streptococcal cell wall-induced arthritis model by either plasmid or local or systemic adminstration of AAV vectors encoding the molecule led to decreased inflammation, pannus formation, bone and joint destruction, and mRNA expression of joint pro-inflammatory cytokines (22). Adenoviral delivery of a soluble TNF receptor type I-IgG fusion protein directly to rabbit knees with antigen-induced arthritis reduced cartilage matrix degradation and decreased leukocyte infiltration into the joint space, especially when administerd in conjunction with a soluble IL-1 type I receptor-IgG fusion protein (14). The above animal model data has led to the initiation of a phase I clinical trial using AAV vectors to deliver soluble TNF Receptor:Fc fusion protein (Targeted Genetics).

1.4.1.3. Inhibitors of IL-18

IL-18 is a pro-inflammatory cytokine that is over-expressed in the synovium of RA patients and correlates with inflammation. Elevated levels of this cytokine are also observed in serum and synovial fluid. Over-expression of an IL-18 binding protein using an adenoviral vector was able to ameliorate arthritis in a CIA model, indicating neutralization of IL-18 may be an effective target in the future treatment of RA (68).

1.4.2. Immune Deviation

An imbalance between Th1 and Th2 cytokines is thought to play a role in the pathogenesis of several inflammatory diseases, including RA. Th1 cells secrete cytokines like IFN-γ that promote a pro-inflammatory environment, while Th2 cytokines secrete cytokines like

IL-4, IL-10, and IL-13 that down regulate Th1 activity. Skewing the immune response from a Th1 response to more of a Th2 response has been suggested as a therapeutic strategy in the treatment of RA. Other strategies aimed at altering the immune response in RA are also discussed.

1.4.2.1. IL-13

IL-13 inhibits activated monocytes/macrophages from secreting a variety of pro-inflammatory molecules. A possible role for this cytokine in the pathogenesis of RA was observed when adenoviral delivery of IL-13 to RA synovial tissues explants led to a decrease in IL-1β, TNF-α, IL-8, MCP-1, NAP-78, PGE_2, and MIP-1α when compared to controls (69). Subsequent studies demonstrated that adenoviral delivery of IL-13 directly to ankle joints in a rat antigen-induced arthritis model significantly decreased paw size, bone destruction, vascularization, inflammatory cell infiltration, and inflammatory cytokine production (70). IL-13 over-expression during immune-complex-mediated arthritis significantly decreased chondrocyte death and MMP mediated cartilage destruction, despite the presence of enhanced inflammation (71).

1.4.2.2. IL-4

Adenoviral delivery of IL-4 to RA synovial tissue explants demonstrated decrease IL-1β, TNF-α, IL-8, MCP-1, and PGE_2 in the cultured medium (72). Intra-articular delivery of adenoviral vectors encoding IL-4 to CIA mice led to an enhanced onset of inflammation but less chondrocyte death and cartilage and bone erosion. Proteoglycan synthesis was enhanced and there was decreased MMP activity (73). IL-17, IL-12, cathepsin K and osteoprotegrin ligand mRNA levels were also reduced (74, 75). Kim *et. al.* observed similar effects on CIA upon local and systemic administration of adenoviral vectors encoding IL-4 (76). AAV-mediated delivery

of IL-4 has also proven beneficial in CIA (55, 56). Electro-transfer of an IL-4 encoding plasmid prior to CIA onset decreased synovitis and cartilage destruction, with an associated decrease in IL-1β in the paw and an increased TIMP2:MMP2 ratio (30). Similar results were seen using either gene gun delivery or intra-dermal administration of plasmid encoding IL-4 (77). Both retroviral and adenoviral delivery of IL-4 in a rat antigen-induced arthritis model had beneficial effects (73, 78). Cell based therapies that deliver IL-4 in arthritis models have also been successful. Injection of fibroblast transfected with plasmid encoding IL-4 decreased histologic evidence of joint inflammation and destruction in a CIA model (79, 80). Likewise, collagen type II pulsed antigen presenting cells engineered to secret IL-4 down-regulated CIA (81).

1.4.2.3. IL-10

IL-10 has demonstrated benefit in several animal models of RA. Either intramuscular administration via electro-transfer or intra-dermal injection of plasmid encoding IL-10 had beneficial effects on CIA (32, 36). Similarly, systemic administration of a plasmid IL-10/liposome mixture decreased signs of CIA after a single intra-peritoneal injection (35).

Viral IL-10 (vIL-10) is homologous to human and mouse IL-10 but, while retaining its immunosuppressive function, lacks many of the immunostimulatory properties of IL-10, and therefore may be a superior treatment option (82). An adenoviral vector encoding vIL-10 was able to decrease CIA when delivered locally or systemically (15, 83, 84). Electrotransfer of viral IL-10 decreased histologic evidence of arthritis in an arthrogen collagen-induced arthritis model and was associated with decreased TNF-α, IL-1β, and IL-6 transcripts in the joint (31). A tet-inducible vIL-10 transgene delivered by AAV was able to decrease macroscopic, radiology, and histologic signs of CIA only when doxycycline was administered (57).

1.4.2.4. TGF-β

TGF-β is a pleiotropic cytokine with many different effects on many different cell types and has been suggested to play a role in RA. While some of its effects, like immunosuppresion, would appear to be beneficial for RA, it has also been associated with pro-inflammatory activity. Not surprisingly, data from animal models using gene transfer support both possibilities, making its true role in RA difficult to decipher. Splenocytes from CIA mice were isolated and infected *ex vivo* with a retroviral vector encoding TGF-β. These cells were then injected in the intra-peritoneal cavity 5 days after arthritis onset. Without TGF-β expression, an exacerbation of arthritis is normally observed. However, TGF-β expressing splenocytes were able to inhibit this exacerbation and also resulted in a decrease in MMP2 activity and a transient reduction in anti-collagen type II antibodies (85). In a rat streptococcal cell wall-induced arthritis model, intramuscular delivery of plasmid encoding TGF-β showed significant decreases in inflammatory cell infiltration, pannus formation, bone and joint destruction, and inflammatory cytokine production (28). In contrast to the reports above, another study found that injection of adenovirus encoding TGF-β into knees of rabbits with antigen-induced arthritis resulted in significant pathology in the knee joint and surrounding tissue, suggesting TGF-β therapy may not be suitable for treating arthritis in some models (86).

1.4.2.5. CTLA-4Ig

CTLA-4Ig fusion protein binds to the co-stimulatory molecules B7-1 and B7-2 present on antigen-presenting cells and blocks CD28/B7 interactions, resulting in decreased T cell activation. It has been shown to ameliorate several experimental autoimmune diseases, including CIA. A single intravenous injection of an adenovirus encoding CTLA-4Ig fusion protein suppressed established CIA as least as efficiently as repeated injections of monoclonal antibody

to CTLA-4. Pathogenic cellular and humoral responses were also diminished in adenoviral vector treated group as compared to antibody treated and control groups (87). CIA could also be inhibited both histologically and clinically by intra-articular administration of a low dose of adenovirus encoding CTLA-4Ig fusion protein (88).

1.4.3. Promoting Apoptosis

One of the key features of RA is the hyper-proliferation of the synovial lining leading to pannus formation, which has been shown to contribute to cartilage invasiveness and bone destruction. Decreasing proliferation of these cells by promoting their apoptosis has been suggested as a treatment strategy for RA (89).

1.4.3.1. TNF-related apoptosis-inducing ligand

In a rabbit model of arthritis, intra-articular adenoviral delivery of TNF-related apoptosis-inducing ligand (TRAIL) was able to increase apoptosis in the syovial cell lining, decrease inflammatory cell infiltration, and promote new matrix depostion (90). CIA mice injected with collagen type II-pulsed antigen presenting cells engineered to express TRAIL under control of a doxycycline inducible promoter decreased the incidence of arthritis and infiltration of T cells in the joint only in the presence of doxycycline. *In situ* TUNEL staining demonstrated TRAIL-induced apoptosis of activated T cells in the spleen (91).

1.4.3.2. Fas ligand

Injection of adenovirus expressing Fas ligand (FasL) into joints of CIA mice induced apoptosis and ameliorated CIA. IFN-γ production by collagen-specific T cells was also reduced (92). *Ex vivo* modified T cells engineered to express FasL were injected into human RA synovial tissue that had been implanted in SCID mice. Analysis of the tissue following treatment demonstrated that synoviocytes and mononuclear cells present in the tissue had been eliminated

by apoptosis through a Fas/FasL interaction (93). Similar results were observed when adenovirus encoding FasL was injected directly into the implanted tissue (94). Dendritic cells modified to by adenoviral vectors to express FasL were able to suppress CIA when systemically injected and also demonstrated decreased IFN-γ production from spleen-derived lymphocytes and decreased T-cell proliferation in response to collagen stimulation (95).

1.4.3.3. Fas-associated death domain protein

Fas-associated death domain protein (FADD) also plays a key role in Fas-mediated apoptosis of synovial cells. It was found that adenoviral vector expression FADD could induce apoptosis in synoviocytes both *in vitro* and *in vivo*, suggesting this strategy may be effective in the treatment of RA (96).

1.4.4. Anti-angiogenis

Hyper-proliferation of the synovial lining is also associated with neo-vascularization in the local environment of the joint. This angiogenesis is necessary for the development and maintenance of the pannus and also provides nutrients required for the survival and proliferation of infiltrating inflammatory cells (97, 98). A peptide targeted to the integrins present in the inflamed synovium and associated with angiogenesis was fused to an anti-apoptotic peptide. Systemic administration of this fusion peptide in a CIA model resulted in decreased clinical arthritis and increased apoptosis of synovial blood vessels (99). 3T3 fibroblasts modified with retroviral vectors to express angiostatin were able to decrease pannus formation and cartilage erosion when injected into knee joints of mice with CIA. Arthritis-associated angiogenesis was also inhibited (100). AAV and HIV vector-mediated delivery of angiostatin to CIA knee joints were similarly beneficial (48, 58). The potent anti-angiogenic factor endostatin was able to

decrease arthritis and reduce blood vessel density in a human TNF-transgenic mouse model of arthritis when delivered to knee joints using a lentiviral vector (47).

Tie2 has also been demonstrated to play a role in angiogenesis in arthritis. Adenoviral delivery of a soluble receptor for Tie2 resulted in a decreased incidence and severity of CIA, inhibition of angiogenesis, and was associated with decreased bone destruction that appeared to result from a decrease in RANKL (101). VEGF is another angiogenic factor that promotes synovitis and bone destruction in arthritis. When soluble VEGF receptor I was administered via adenoviral vectors to CIA mice, disease activity was suppressed significantly (102). Thrombospondins (TSP1 and 2) have also been shown to inhibit angiogenesis and also decrease pro-inflammatory cytokine production in animal models of arthritis (103, 104). In addition, adenoviral gene transfer of a urokinase plasminogen inhibitor was also able to inhibit angiogenesis in a CIA model (105).

1.4.5. Targeting Matrix Degradation Enzymes

Matrix metalloproteinases (MMPs) degrade extra-cellular matrix components and have been demonstrated to contribute to cartilage degradation in RA. Ribozymes and an anti-sense construct targeting the destruction of MMP-1 delivered to RA synovial fibroblasts via retroviral vectors decreased MMP-1 production and reduced the invasiveness of the fibroblasts in a SCID mouse model of RA, suggesting this may be an effective approach to inhibiting cartilage destruction in RA (106, 107).

Another strategy that has proven successful is to increase the ratio of tissue inhibitors of matrix metalloproteinases (TIMPs) to MMPs. In a rat antigen-induced arthritis model, intramuscular injection of naked DNA encoding TIMP-4 completely abolished the development of the disease (33). Likewise, adenoviral delivery of TIMP-1 and TIMP-3 to RA synovial

fibroblasts significantly decreased their invasiveness both in vitro and in an in vivo SCID mouse model of RA. These molecules were found to act by both decreasing MMP production and reducing cell proliferation (108).

1.4.6. Targeting NFκB

The transcription factor NFκB plays a significant role in the activation of many cytokines that contribute to the pathogenesis of RA. As such, inhibition of this factor could lead to therapeutic benefit in RA. Injection of decoy oligodeoxynucleotides with high-affinity for NFκB into ankle joints of CIA rats significantly decreased joint swelling and joint destruction. The levels of the pro-inflammatory cytokines TNFα and IL-1β were also decreased in the treated joints (109). Another group found that inhibiting NFκB in RA synovial fibroblasts using an adenovirus to deliver a dominant negative inhibitor of NFκB led to an increase in apoptosis upon stimulation with TNFα. These cells are normally resisitant to apoptosis when stimulated with TNFα, suggesting that this strategy may be beneficial in the treatment of RA (110).

1.4.7. Other candidate genes

Other molecules that have been demonstrated to play a role in arthritis using gene transfer in various *in vitro* or animal models are Csk (inhibitor of Src activity that suppresses bone destruction in rat adjuvant arthritis), cathepsin L (targeted destruction of cathepsin L by ribozymes is beneficial in SCID/RA tissue coimplantation model), fibronectin (inhibits leukocyte infiltration and suppresses inflammation in CIA), galectin-1 (suppresses CIA via T cell apoptosis), IFN-β (ameliorates CIA by suppressing IFN-γ, TNF, and IL-12 production), p16INK (induction of p16INK in rat adjuvant arthritis prevented pathology), p21 (adenoviral delivery ameliorated rat adjuvant arthritis), SOCS3 (adenoviral delivery of SOCS3 was beneficial in CIA), soluble CR1 (delivery by retrovirally infected syngeneic cells or naked DNA suppressed

CIA), superoxide dismutase and catalase (amelioration of antigen-induced arthritis in rats and CIA in mice), and Ras (dominant negative ras suppresses bone destruction in rat adjuvant arthritis). (29, 34, 111-120)

1.5. Future of Gene Therapy for Arthritis

Much progress has been made in the past several years in the use of gene therapy for the treatment of arthritis. However, there is much room for improvement. Future studies will look at improving targeted delivery of vectors, regulating transgene expression, and improving the safety and efficacy of the vectors already in use. Results from clinical trials will hopefully solidify the idea that gene therapy is the future in the treatment of RA.

1.6. AAV biology

1.6.1. AAV structure and genome

Adeno-associated virus is a small, non-enveloped, single-stranded DNA virus with broad tissue tropism. It is a non-pathogenic virus belonging to the *Parvoviridae* family and has a 4.68kb genome. In human and non-human primate tissues eleven different serotypes of AAV (1-11) have been isolated and characterized. Serotypes are defined by the inability of antibodies against a specific viral capsid to neutralize the viral capsid of another serotype. If a specific isolate can be neutralized by existing serum to a serotype but still contains differences within the sequence of the viral capsid, it is considered a sub-type or variant of that serotype. In addition, mutations can be made to an existing viral capsid, altering the ability of existing antibodies to neutralize the virus. Many of these mutants have not been tested against a panel of antibodies to all known serotypes and are generally termed hybrid serotypes. AAV has also been isolated from several other species, including cow, chicken, sheep, snake, lizard, and goat (121). Of the eleven serotypes, AAV2 is the best characterized.

Wild type AAV normally requires a helper virus such as adenovirus or herpesvirus to produce active infection. The viral genome consists of two viral genes, *rep* and *cap*, flanked on both sides by a 145 base pair inverted terminal repeat (ITR). The viral ITRs are the only *cis*-acting elements necessary for viral replication and packaging. Four rep proteins are necessary for viral replication (Rep78, Rep68, Rep52, and Rep40). Two promoters and alternative splicing are used to generate the viral Rep proteins. Another promoter drives expression of the three viral capsid proteins (VP1, VP2, and VP3), which are made through the use of alternative splicing and alternative translation start codons. The three proteins are combined in a ratio of 1:1:20 of VP1, VP2, and VP3, respectively, to generate a virus with an icosahedral capsid that is 26nm in diameter (122, 123).

1.6.2. Site-specific integration

In the absence of helper virus, latent infection can be established by site specific integration into the AAVS1 locus of human chromosome 19 (124, 125). The host and viral requirements for integration have been defined. A 34bp sequence on chromosome 19 is sufficient to mediate integration (126, 127). For the virus, Rep protein expression and a sequence in the p5 promoter region are required (128-130). Integration has been associated with duplications, deletions, and amplification at the AAVSI locus (131, 132).

Although site-specific integration has been observed in cultured cells, no study has ever demonstrated site-specific integration in human tissues. There is one report of an integration event in the AAVS1 locus in non-human primates (133). In fact, it appears that wild type AAV (similar to recombinant AAV) exists mainly in circular double-stranded episomes in *in vivo* tissue samples. Out of 175 tissues tested (tonsil, spleen, and lung), only 9 tested positive for AAV. No samples were found to have integration of the AAV genome into chromosome 19.

Only one sample demonstrated an integration event, which was in chromosome 1 (134). A large scale *in vitro* analysis in normal human fibroblast cells using a shuttle vector system to identify vector-chromosome integration junctions identified many hot spots for AAV integration in the human genome, including chromosome 19 (135). Portal vein injection of AAV, however, did not appear to predispose mice to tumor formation in a large scale study (136). Another study in HeLa cells revealed that at a multiplicity of infection (MOI) of 100, 80% of cell lines had AAVSI rearrangements and half of those had integrants. Virtually all integrants appeared to be site-specific (132). Regardless, there is conflicting literature about the frequency and nature of the integration of wild-type AAV both *in vitro* and *in vivo*.

1.6.3. Recombinant AAV vectors

Recombinant AAV vectors have been developed for use in gene transfer studies. Vectors are typically produced by the 293 triple transfection method. 293 cells are transfected with three plasmids. One plasmid contains the *rep* and *cap* genes and another contains a transgene flanked by viral ITRs. The viral ITRs contain the signals necessary for packaging of the transgene into the viral capsid. The third plasmid encodes the adenoviral genes necessary for helper virus function. The virus is then harvested from the cells and then purified. The resulting virus is replication incompetent and carries the transgene of choice.

The packaging limit for a single AAV virion is about 5kb, which hinders the ability to use AAV in gene transfer studies, as many genes are much larger than this. However, trans-splicing, in which a gene is split into two and tagged with splice donor and acceptor sites, has recently become more efficient and expanded the capacity of AAV to 10kb. Following co-infection, viral genome recombination and splicing allow a full-length gene product to be made. Using this method, both adult and aged *mdx* mice, which have a dystrophic phenotype,

demonstrated amelioration of the disease following delivery of the 6kb mini-dystrophin gene to skeletal muscle (137). This approach may help to expand the diseases that recombinant AAV vectors can be used to treat.

Because recombinant vectors lack the *rep* gene, site-specific integration does not occur. Instead, long-term expression appears to be mediated by circular episomal concatamers of double-stranded viral DNA (138). In animal studies, transgene expression from AAV2 vectors has been observed for up to 6 years in rhesus macaques following intramuscular administration (139).

Random integration events into the host chromosome are always of concern in the field of gene therapy because of the possibility of disruption of potential oncogenes, which could lead to the development of cancer. Therefore, determining the frequency of its occurrence is of paramount importance if a vector is to be successfully used in gene therapy.

The relative contribution of episomal and integrated AAV vector genomes to stable transgene expression was examined in an *in vivo* mouse model. Mice were infected with recombinant AAV vectors by portal vein injection, followed by a 2/3 partial hepatectomy 6 weeks to 12 months later. If the vector had integrated stably, transgene expression would be expected to have returned to levels present prior to surgery after subsequent hepatocellular regeneration. However, in this study transgene levels were reduced by 90%, indicating that episomal forms of the vector are primarily responsible for long-term hepatocyte expression *in vivo* (140). Nonetheless, the AAV viral ITRs do appear capable of playing a role in random integration. Adenovirus/AAV hybrid vectors have been developed in which the AAV ITRs flank the transgene. These vectors were demonstrated to integrate randomly in the genome in a AAV ITR-dependent manner (141, 142). Although random integration is not thought to occur with

recombinant AAV vectors, this study suggests that under the right circumstances (i.e. adenovirus co-infection) it may be possible.

1.6.4. Clinical trials with AAV

At least 25 clinical trials using recombinant AAV have been completed, initiated, or proposed. Diseases under study include hemophilia, cystic fibrosis, Parkinson's disease, Alzheimer's disease, Canavan disease, and arthritis, among others (143). To date, all trials have utilized AAV2, although future studies are likely to include other serotypes.

1.6.5. AAV tropism

The first step in AAV infection is viral binding to the cell surface via high affinity cellular receptors. Different serotypes of AAV infect different cell types based on the availability of their respective receptors and co-receptors. This is demonstrated by the wide variability in the tropism of the different serotypes of AAV in different cell types and in different species. Several examples are discussed below.

1.6.5.1. Intraspecies variation

AAV6 gave more efficient transduction of mouse epithelial cells in both large and small airways than AAV2 (144). When injected into day 15 fetuses *in utero*, AAV5 encoding luciferase provided higher expression than AAV2 when the mice were examined 15 months after birth (145). Following sub-retinal injection in mice, AAV5 proved more efficient in transducing photoreceptor cells than AAV2 (146). AAV2, AAV4, and AAV5 demonstrated different tropism after injection into the mouse central nervous system. AAV4 and 5 gave 10-fold more transgene positive ependymal cells than AAV2 afer injection into the lateral ventricle of mice. After striatal injection, AAV4 demonstrated mainly ependymal cell transgene expression, but no expression was detected in the parenchyma. In contrast AAV2 and 5 both had transgene

expression in parenchymal cells following striatal injection, with AAV2 transgene expression confined mainly to neurons, while for AAV5 transgene expression was detected in both neurons and astrocytes. In addition, 15 weeks after injection, AAV5 gave 5000-fold higher expression than AAV2 (147).

There were distinct patterns of transduction in the gerbil hippocampus using either AAV2 or AAV5, with AAV2 infecting pyramidal and granular cells and AAV5 infecting primarily granular cells (148). Differential transduction and cell tropism was also observed after injection of AAV serotypes 1, 2, and 5 into different regions of the rat brain (149). AAV3 could infect human myeloid and megakaryocytic cells that were resistant to infection with AAV2 (150).

A thorough examination of AAV2 transduction in primary human cells demonstrated significant cell-specific differences in transduction and duration of transgene expression. In particular, skeletal muscle and hair-follicle cells appeared suited for long-term expression of AAV. In some cell types, expression improved over time. In other cell types, expression went down over time. There was an inverse relationship between long-term trangene expression and the proportion of cells in the G2/M phase, consistent with the idea that most of the viral genome is present as episomes that are diluted out between cells as they divide (151).

There was also significant donor variation in AAV2 transduction in bone marrow derived CD34-positive cells of healthy volunteers. There was significant variability in transgene expression depending on the level of cellular differentiation. This has implications for the use of AAV in gene therapy involving hematopoietic stem/progenitor cells (152).

1.6.5.2. Interspecies variation

Mouse and human airway epithelia demonstrate species-specific differences in AAV transduction, highlighting that the serotype of AAV best suited for animal model studies may not

be the same serotype that will work best in human and nonhuman primate models. AAV2 and AAV5 gave approximately equal transduction of human airway epithelia, while AAV5 gave much higher transduction than AAV2 in mouse lung (153). Cross-packaging of AAV2 vector genomes with viral capsids from different serotypes demonstrated that the resulting viruses enabled transduction with broad and differing specificity. In addition, it was readily apparent that different target tissues of rats and mice had a different hierarchy of serotypes for efficient transduction (154).

1.6.6. Viral Receptors

Membrane associated heparan sulfate proteoglycan (HSPG) is the primary attachment receptor for AAV2, although other proteoglycans may be involved as well (155, 156). Fibroblast growth factor receptor 1 (FGFR1), $\alpha_v\beta_5$ integrin, and the hepatocyte growth factor receptor c-Met have all been identified as co-receptors for AAV2 (157-159). Receptors for some other AAV serotypes have also been identified (Table 1). Unlike AAV2, AAV4 and AAV5 are insensitive to heparin competition, suggesting they enter cells via a distinct receptor. Both viruses have been demonstrated to bind to sialic acid, although the tropism of these viruses is distinct. For AAV5, binding to α2,3-linked sialic acid is required for efficient transduction (160). Kaludov et. al. demonstrated that α2,3 O-linked sialic acid is required of AAV4 binding, while α2,3 N-linked sialic acid is required for AAV5 binding, could account for the differences in tropism between these two viruses (161). Platelet derived growth factor receptor (PDGFR) has also been identified as a cellular receptor for AAV5 (162). The remainder of the discussion on the infectious entry pathway of AAV will focus on AAV2.

Table 1. Receptors for Different Serotypes of AAV.

Virus	Receptor	Co-receptor
AAV2	Heparan Sulfate Proteoglycan	$\alpha_v\beta_5$ integrin, fibroblast growth factor receptor type 1 (FGFR), and hepatocyte growth factor receptor (HGFR)
AAV4	α2,3 O-linked sialic acid	
AAV5	α2,3 N-linked sialic acid, α2,6-linked sialic acid	PDGFRα

1.6.7. Viral entry and intracellular trafficking

1.6.7.1. Receptor-mediated endocytosis

Entry of AAV into HeLa cells is dependent on dynamin, a GTPase involved in the clathrin-mediated internalization of plasma membrane receptors and their ligands, indicating that the primary pathway of AAV entry into cells is endocytosis via clathrin-coated pits (163, 164). Internalization of AAV2 also requires activation of the small GTP-binding protein Rac1. Subsequent activation of PI3K is necessary to initiate intracellular trafficking of AAV2 to the nucleus via microfilaments and microtubules (165).

1.6.7.2. Endosomal Trafficking

Once the virus enters the cell via endocytosis, the exact nature of its path to the nucleus is somewhat controversial. Bafilomycin A1 is a specific inhibitor of the vacuolar protein pump that inhibits acidification of the endosome. In HeLa and 293 cells, the presence of bafilomycin A1 significantly impaired AAV transduction, demonstrating the importance of the acidification of the late endosome for viral escape into the cytoplasm (164, 166). Likewise, ammonium chloride, which increases the pH of intracellular organelles, prevented gene transduction when

present during the first 30 minutes after endocytosis, again demonstrating a requirement for an acidic environment for escape into the cytoplasm (164). However, in the glioma cell line LN-229, which, like HeLa and 293 cells, is highly permissive for AAV transduction, bafilomycin A1 had little effect. This is despite the fact that viral localization within the cell appears identical to that of HeLa and 293 cells, with perinuclear localization occurring by 1hr after infection (167). This suggests that in glioma cells the infectious entry pathway is independent of late endosomal acidification. It should be noted that within 30 minutes after infection in HeLa and LN-229 cells some virus can also be seen in the trans-Golgi, an effect that was seen only at low multiplicity of infections (MOIs) (167). AAV5 has also been observed to traffic to the trans-Golgi in HeLa cells (168).

In another study in HeLa cells, AAV was observed to enter the cell rapidly and localized to early endosomes. The virus then escaped quickly from the early endosomes and entered the cytoplasm, where it quickly accumulated perinuclearly (169). Real time single molecule imaging of Cy5 labeled AAV particles in HeLa cells also revealed that virus was endocytosed quickly and became localized in endosomes. The virus moved in a directed fashion towards the nucleus dependent on the microtubule motor proteins kinesin and dynein, although a significant fraction of virus was also found to be free-floating in the cytoplasm shortly after infection (170).

To clarify these somewhat contradictory data, further studies using imaging and sub-cellular fractionation techniques were performed to examine the intracellular trafficking of AAV2 in HeLa cells in further detail. AAV2 was found to be directed to either late endosomes or recycling endosomes in a dose-dependent fashion. At low MOI, most of the AAV could be found in the late endosomal compartment. At higher MOIs, most of the virus was found in

recycling endosomes (171). This suggests that the route of viral trafficking is dependent on the amount of virus that enters the cell.

The amount of virus entering each cell would be dependent on several factors, the most important of which are the level of viral receptor on the cell surface and the rate of endocytosis. Differences in receptor levels and endocytosis rates, therefore, could account for the differences in viral trafficking observed between different cells infected at the same multiplicity of infection (i.e. HeLa and LN-229 cells). The importance of endocytic processing of AAV is highlighted by studies of AAV infection of mouse 3T3 fibroblasts, which are less permissive for AAV transduction. In these cells, it has been demonstrated that virus fails to enter the nucleus due to altered endocytic processing (172). In fact, direct labeling and tracking of single-stranded AAV genomes using BrdU demonstrated that intracellular trafficking, not second-strand synthesis, appeared to be rate-limiting for AAV transduction in HeLa cells (173). Differences in endosomal processing and nuclear trafficking of AAV2 were also observed in human airway epithelia depending on whether it was internalized on the apical or basolateral surface (174).

In HeLa cells, both AAV2 and AAV5 capsids appear to be substrates for ubiquitination and subsequent degradation by the proteasome. However, it appears that the capsid must first be modified in the endosome before it can be conjugated with ubiquitin, as intact virions are not substrates for ubiquitination *in vitro*. The exact nature of this modification remains unknown (175). The proteasome inhibitor zLLL increased transduction in HeLa cells up to 50-fold, resulting in an accumulation of viral genomes within the cell (166).

1.6.7.3. Nuclear trafficking

Following escape into the cytosol, AAV accumulates perinuclearly as early as 15-30 minutes after induction of endocytosis (164, 170). In general, translocation into the nucleus

appears to be a slow process. Most of the intact virus was observed in a peri-nuclear location and within nuclear invaginations but not in the nucleus. Viral genomes, meanwhile were readily visible within the nucleus, suggesting that uncoating probably occurs before or during nuclear entry (176). However, there are reports of intact virus entering the nucleus prior to uncoating (164). This may be possible because the nuclear pore size is approximately the same as the diameter of AAV, which may allow for diffusion of some intact virus across the nuclear membrane. In general, transport of virus into the nucleus appears to be an active process, that like trafficking within the cytoplasm, is dependent on dynein and kinesin. Although microtubules and their associated motor proteins were not previously known to be present in the nucleoplasm, it has been demonstrated that tubular structures formed by invagination of the nuclear membrane exist and even transect the nucleus. Because the core of these channels is continuous with the cytoplasm, it is possible that microtubules could polymerize within them (170).

In the presence of adenovirus intact AAV particles can rapidly enter the nucleus soon after infection. Nuclear transport in the presence of adenovirus appears to be independent of the nuclear pore complex (NPC), as the NPC inhibitor thapsigargan had no effect on translocation of AAV to the nucleus (169). This is consistent with studies using intact, purified nuclei from both permissive and less permissive cell types in which AAV was observed to enter nuclei in a time and temperature dependent manner. This process was also independent of the nuclear pore complex and intact virus could enter the nucleus. The purified nuclei appear to have all the machinery necessary for uncoating and second-strand synthesis (177).

Uncoating itself must not be understated, however, as in mouse liver, AAV transduction appears to be limited by the rate of uncoating of the virus particles in the nucleus (178). The

VP1 capsid protein contains phospholipaseA2 (PLA2) activity that is required for efficient transduction of HeLa cells. Mutations in this region do not affect viral packaging, cell binding or entry, but appear to affect a step after perinuclear accumulation of the virus but prior to early gene expression, perhaps uncoating (179).

Once the viral genome is in the nucleus, second-strand synthesis must occur before transcription and subsequent gene expression can occur. The AAV ITR contains a self-priming mechanism, which, in combination with cellular factors, facilitates second-strand synthesis and results in a double-stranded template that is nicked on one end. Several genotoxic agents have been shown to increase AAV transduction by increasing second-strand synthesis, suggesting that this is a rate-limiting step in AAV transduction (180, 181). Self-complementary or double-stranded AAV vectors, which bypass the need for second-strand sythesis, have been developed and provide for rapid and efficient transduction in several cell types both *in vitro* and *in vivo* that is superior to single-stranded vectors (52, 182, 183). Self-complementary duplex strand AAV vectors have also demonstrated better transgene expression than single-stranded vectors in mouse muscle *in vivo*, especially at early time periods (184).

Although second-strand synthesis has been suggested to be rate-limiting for stable and persistent expression from recombinant AAV vectors *in vitro* and *in vivo*, there is evidence that complementary strands can also be generated not by synthesis but by intracellular recruitment of plus and minus single stranded AAV genomes. It appears that these forms may be primarily responsible for stable *in vivo* transduction and that intermolecular joining, not genome amplification, is responsible for concatemer formation in hepatocytes *in vivo* (185).

1.6.7.4. Summary

The pathway of AAV infection has been examined in many cell types by several different methods. Many factors, including MOI, cell type, receptor expression, etc., appear to affect the pathway of AAV infection. The data gathered to date are summarized below (Figure 1). In brief, AAV binds to it primary receptor (HSPG) and co-receptor on the cell surface and is internalized by receptor-mediated endocytosis via clathrin-coated pits. Rac1 and PI3K activity are required for intra-cellular movement towards the nucleus. Viral movement within the cell is dependent on microtubules. At low MOI, most of the virus traffics to the late endosomes. At high MOI, virus preferentially traffics to the recycling endosomes. There is evidence that virus can escape from early, late, and recycling endosomes. The primary signal for escape into the cytoplasm appears to be acidification of the endosome, although other factors may play a role. Once the virus escapes into the cytoplasm it becomes ubiquitinated and can be degraded by the proteasome. Presumably, some virus evades the proteasome and traffics to the nucleus by a mechanism that appears to be independent of the nuclear pore complex. The virus becomes uncoated, most likely during nuclear transport, a process that is also dependent on the microtubule network of proteins. Once the viral genome is within the nucleus, a double-stranded form of the genome must be generated, either by second strand synthesis or binding to a complementary stranded genome, before transcription can occur.

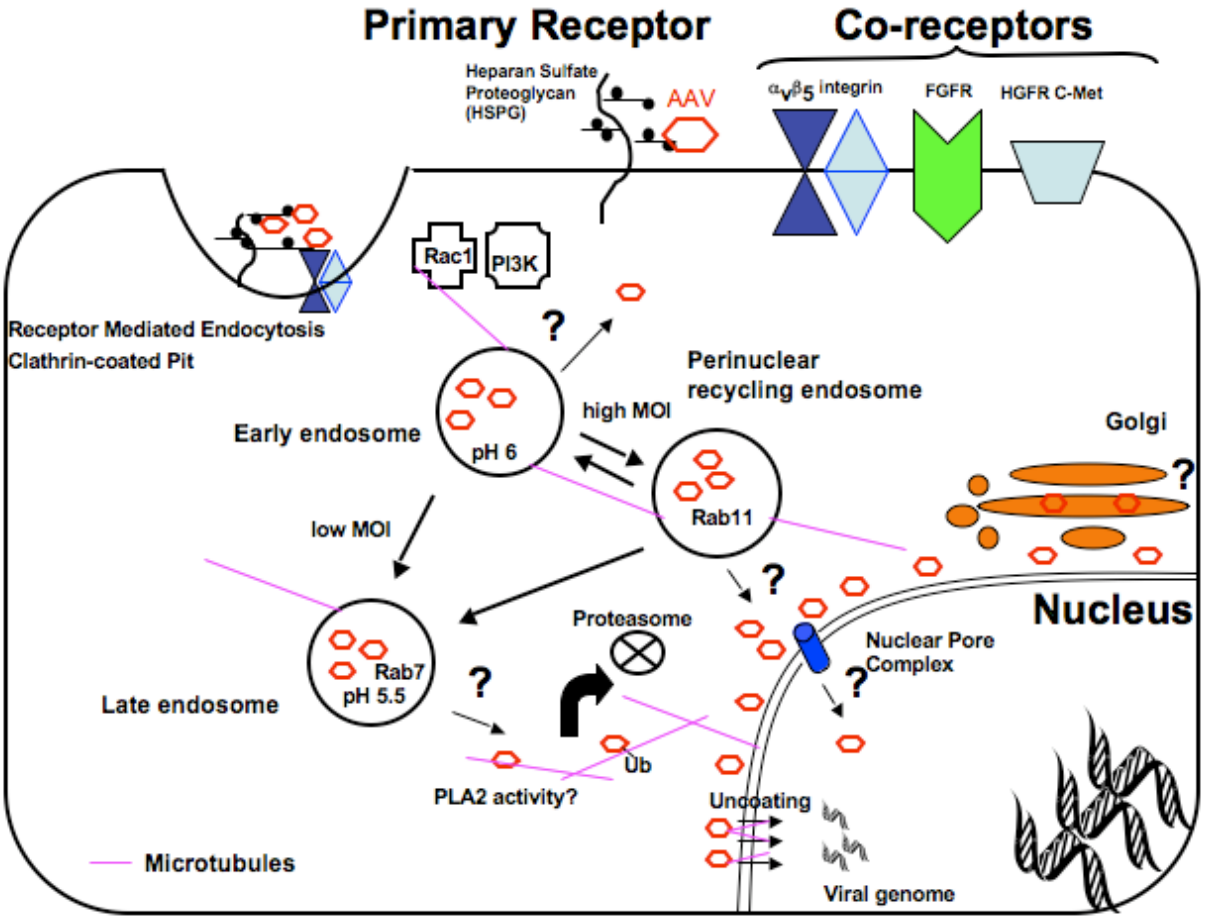


Figure 1: Summary of the pathway of AAV infection.

1.6.8. Antibodies to AAV

1.6.8.1. Prevalence of antibodies to AAV

One concern about the use of viral vectors in gene therapy is the formation of neutralizing antibodies to the vector, which could prevent successful administration or re-administration. This is especially a concern for AAV, because although it is a non-pathogenic virus, seropositivity is widespread. Anti-AAV antibodies were found in 80% of normal human subjects, while 18% had neutralizing antibodies (186). In another study, anti-AAV antibodies were present in 96% of patients, with 35% having neutralizing antibodies (187). Antibodies are present in populations across Europe, Asia, and North America, indicating worldwide infection.

The presence of anti-AAV antibodies peaks in children and subsequently declines. Another peak occurs after age 30. Interestingly, pregnant women also have a higher titer of anti-AAV antibodies (188).

1.6.8.2. Effects of AAV antibodies on transduction

Several studies have been performed to examine the effect of the presence of anti-AAV antibodies on successful AAV transduction. Passive human antibody administration (pooled intravenous immunoglobulin samples) to SCID mice was able to prevent liver transduction by AAV at even very low titers, suggesting that alternative routes of administration or use of different AAV serotypes from primate or non-primate sources may be necessary if repeated administration of the vector is desired (189). The feasibility of this approach was tested in the rat brain where presence of circulating antibodies to wild-type AAV2 could prevent successful gene transfer using recombinant AAV2, but did not prevent recombinant AAV5-mediated transduction (190). Another possibility is the use of polyethylene glycol to coat the capsid (PEGylation), which has been demonstrated to prevent neutralization of adenovirus and allow transgene expression upon readministration of the vector. Using tresyl chloride PEGylation of AAV, levels of transgene expression were comparable to naïve animals after intravenous readministration of virus, but not after readministration intramuscularly. Therefore this approach may be most successful for AAV transgenes targeted to the liver (191). Another approach is to alter the capsid itself so it is resistant to neutralization. In fact, mutations in the AAV2 capsid have been identified that lead to reduced affinity and neutralization effects of anti-AAV serum antibodies (186, 192).

There are several factors that appear to influence the exact nature of the immune response to AAV vectors. The route of administration determined the T-cell independent humoral

responses to AAV vectors in mice (193). In another study, non-human primates were injected with wild type AAV intramuscularly, intravenously, or intranasally. Only animals injected intramuscularly or intravenously developed a humoral response, although co-infection with helper adenovirus led to a humoral response when administered intranasallly along with AAV. Cell-mediated responses to the AAV capsid were only seen in the presence of helper adenovirus (133).

Neutralizing antibodies to AAV persisted for up to one year following intramuscular administration of the vector in mice and non-human primates. The antibody response was T-cell dependent. Re-administration of AAV led to a 4-20 fold reduction in transgene levels in the treated animals. However, this diminution could be effectively prevented by administering a CD4 T cell blocking antibody at the time of the first injection, presumably by avoiding activation of memory B cells (194). In contrast, when AAV is delivered to the liver by intra-portal infusion after the mice were initially immunized with AAV by intravenous injection, no transgene expression could be detected, even if the mice were immunosuppressed with anti-CD40 or anti-CD4 antibodies prior to re-administration of the virus (186). These results also suggest that the route of administration has an effect on the nature of the antibody response to the virus.

In the rabbit airway, repeated delivery of AAV2 continued to result in significant transgene expression, despite high titers of neutralizing antibodies in the serum (195). This is in contrast to another report in which transduction of rabbit airway was minimal and the presence of neutralizing antibodies correlated with an inability to successfully re-administer the virus (196). This difference has been attributed to possible airway trauma because of the technique used or augmentation of the immune response due to contaminating wild type AAV. In the mouse airway, if anti-CD40 ligand antibody and a soluble CTLA4-immunoglobulin fusion

protein were given at the time of primary exposure to the AAV vector, neutralizing antibody production was prevented and the vector could be successfully re-administered (197).

1.6.8.3. Immune response to the transgene

Lastly, the development of an immune response against the transgene itself must also be considered. The immune response to AAV vectors may also depend on the tissue in which the transgene is expressed. The use of AAV vectors with liver-specific promoters appeared to increase transduction in murine liver and decrease formation of neutralizing antibodies to the transgene. In a murine model of Fabry disease, mice treated with these vectors developed tolerance to the transgene, human α-galactosidase A. These mice expressed the transgene at therapeutic levels for up to a year (198).

1.6.8.4. Summary

The studies described above demonstrate the complexity of the immune response to AAV vectors. The route of administration appears to be the main determinant of the response to the vector. However, modifications to the virus (i.e. by mutations) or modulation of the immune response with other agents show promise in ensuring the successful use of AAV in gene therapy.

1.6.9. Controlling expression from AAV vectors

For many gene transfer applications, it is desired to have expression of the transgene only in certain tissues. In other instances regulated expression is important. Both examples are discussed below.

1.6.9.1. Cell type specific promoters

Tissue-specific expression can be achieved with the use of tissue-specific promoters. For example, injection of AAV2 with neuron-specific enolase or PDGF-beta chain promoters led to neuron specific trangene expression in the rat spinal cord, with an absence of detectable glial cell

transduction (199). Use of a PDGF-beta chain promoter led to more expression in dopaminergic neurons and their projections in the rat striatum than using the conventional CMV promoter. Incorporation of the woodchuck post-transcriptional regulatory element (WPRE) also increased transduction (200). AAV2 with expression driven by a myelin-forming promoter demonstrated oligodendrocyte-specific transgene expression both *in vitro* and *in vivo*. After stereotactic injection into mouse brains, expression in oligodendrocytes was primarily confined to the white matter (201, 202).

1.6.9.2. Regulated expression

AAV vectors with regulated expression have several advantages over traditional AAV vectors. For diseases with a recurring/remitting course, expression can be induced during active disease and repressed during remission. Regulated expression also helps to prevent any deleterious side effects that may result from continuous expression of the transgene.

In a murine model of β-thalesemia, mice receiving intramuscular injections of AAV encoding erythropoietin with expression driven from the constituitive CMV promoter developed polycythemia, leading to decreased survival. Only using an AAV vector with expression regulated by administration of the small molecule drug rapamycin could improvements be observed. Still, the animals required a strict titering of rapamycin levels to achieve optimal levels of erythropoietin (203). This highlights the importance of regulation of transgene expression when using gene transfer vectors.

Tetracycline regulated AAV vectors have also been developed. These vectors can either be designed to be turned off in the presence of tetracycline, or be turned on. AAV vectors designed to be turned off in the presence off tetracycline successfully transduced HeLa cells *in vitro* and rat brains *in vivo*, with large increases in transgene expression observed only in the

absence of the antibiotic (204). Similar success was attained following intramuscular co-injection of AAV vectors in mice, one encoding erythropoietin and the other encoding the tetracycline transcriptional activator (205).

The main problem with vectors that are turned off in the presence of tetracycline is the requirement for the constant presence of antibiotic when it is desired to turn transgene expression off. Although tetracycline is relatively safe, long-term pharmacologic effects of the drug are a concern. Comparatively, vectors that are turned on in the presence of tetracycline may be more applicable in the clinical setting, especially for diseases that require periods of repeated and intermittent treatment, such as neurodegenerative diseases or RA. In a rat model, stereotactic injections of this "tet-on" AAV vector into the rat brain led to an 80-fold induction of transgene expression upon addition of doxycycline to the diet. Although removal of the antibiotic resulted in a rapid decrease in transgene expression (3 days), induciblilty after readministration of the antibiotic took up to 14 days, indicating this vector may not be suitable for diseases in which rapid on/off switching of transgene expression is desired (206).

Lipopolysaccaharride (LPS) also appears to regulate AAV transgene expression. In a rat model of arthritis induced by LPS, it was observed that the inflammation associated with LPS administration significantly and repeatedly enhanced AAV transgene expression in the synovium. Regulation at the level of the CMV promoter, which also drives expression from our AAV vectors, has been implicated in this process (207).

Similarly, in a rabbit model examining AAV transduction of the corneal endothelium, inducible and regulated transgene expression was observed. Upon injection into the ocular anterior chamber of the eye, AAV (with transgene expression controlled by a CMV promoter) demonstrated very little corneal endothelial cell transduction. However, upon administration of

LPS, expression increased dramatically. Upon resolution of inflammation, transgene expression returned to baseline. A second injection of LPS again resulted in a significant increase in transgene expression in the corneal endothelium (208).

1.6.10. Role of the AAV ITR in transgene expression

The AAV ITRs have also been demonstrated to play a role in driving transgene expression. A baculoviral vector with a neuron specific promoter was modified to include AAV inverted terminal repeats (ITRs) flanking a luciferase expression cassette. The resulting vector increased transgene expression in the rat brain from only transient expression to expression lasting longer than 90 days (209). Flanking expression cassettes with AAV ITRs led to increased expression following injection into developing *Xenopus laevis* embryos (210). Injection of genes flanked with AAV ITRs into one-celled zebrafish embryos also resulted in uniform gene expression in the F0 generation and stable transmission to subsequent generations (211). The ITR by itself can also initiate mRNA expression from viral vector templates. The region responsible has been mapped to a 37-nucleotide stretch in the A/D elements of the ITR. This residual transcription may also limit the use of regulated expression cassettes for controlled expression from AAV vectors *in vivo* (212).

1.6.11. Modifying the tropism of AAV by modifying the capsid

AAV can infect a wide range of cell types at varying efficiency. Ideally, AAV vectors could be modified so that directed delivery of genes can be achieved. However, viral receptors like HSPG are widespread and therefore make targeted delivery difficult. In contrast, other cell types lack viral receptors and are resistant to infection. The main viral proteins responsible for binding to cellular viral receptors are the capsid proteins. This virus-cell interaction is the first step of viral infection and is necessary for efficient transgene delivery to the cell. Modification

of the viral capsid has proven important for targeting of AAV to nonpermissive cells, tissues, and organs. The biggest obstacles to this approach have been that capsid mutations often affect viral particle assembly, uncoating of the virus, and/or viral infection rates.

The region of the capsid necessary for heparin binding has been mapped to regions around 509-520 and 580-590. Heparin binding was completely eliminated by insertion of an RGD-4C peptide into two locations in the capsid (at positions 520 and 584). The resulting virus contained novel tropism and could infect cells expressing the cellular integrin receptor, to which the RGD epitope binds (213, 214). Similarly, others found that mutations to R585 and R588 of the capsid eliminated heparin binding (215). Another study defined several capsid mutations that were important for viral transduction and antibody neutralization (216). By inserting a single chain antibody for CD34, a cell surface marker for hematopoietic stem/progenitor cells, into the AAV capsid, a CD34-positive human myoleukemia cell line previously refractory to AAV infection could be efficiently transduced (217). A targeting peptide, L14, which is able to recognize an integrin receptor, was inserted into the capsid, allowing transduction of cells previously resistant to AAV2 infection (218). Another group was able to introduce mutations in the capsid that introduced both affinity for the NMDA receptor and cytoplasmic dynein, allowing both targeting to neuronal axons and facilitating retrograde transport of the virus, providing an efficient and noninvasive method for introducing transgenes into the brain for treatment of neurological disorders (219). AAV mosaics incorporating Protein A into their capsid, along with targeting antibodies, allowed specific transduction of cell types previously resistant to AAV transduction (220).

Endothelial cell targeting peptides were identified by phage display and subsequently inserted into the AAV capsid. The resultant virus specifically targeted endothelial cells both *in*

vitro and *in vivo* (221). Likewise, a insertion of a peptide specific for the human luteinizing hormone receptor targeted ovarian cancer cells in an HSPG-independent manner (222). Introducing a ligand for the low-density lipoprotein receptor (LDL-R) into the capsid of AAV2 allowed targeting to murine islets *in vitro* and hepatocytes *in vivo*, both of which express LDL-R (223).

Using a combinatorial approach to insert random peptides into the capsid at a site known to be amenable to insertions, it was possible to create AAV vectors that could infect cells previously resistant to AAV infection and independent of the wild-type viral receptor, HSPG (224). If AAV is to become a widely used gene transfer vector, this approach highlights the wide adaptability of these vectors for specific targeting to many different cell types.

Targeting of AAV vectors to cells resistant to transduction has also been possible by taking advantage of the high affinity interaction of streptavidin and biotin. The AAV capsid could be labeled with biotin without affecting its natural tropism. By incubating the virus with targeting peptide/streptavidin fusion proteins, the virus could be efficiently targeted to cell types that were previously resistant to AAV transduction, while still retaining natural tropism as well (225).

Modifying the AAV2 capsid by inserting an immunoglobulin binding domain, followed by coupling to various antibodies, could efficiently re-direct AAV2 to cell types previously not permissive for AAV transduction (226). Insertion of tumor targeting peptides into the AAV capsid was able to alter the tropism of the virus to rhabdomyosarcoma cells expressing a receptor for the peptide (227). Likewise, insertion of the serpin receptor ligand into the N-terminus of VP1 or VP2 could alter the tropism of the virus (228). Megakaryocyte cell lines non-permissive for AAV transduction could be targeted with the use of a bispecific Fab antibody, with one arm

specific for the AAV capsid and the other for an integrin present on the megakaryocyte lines. Importantly, the targeting substantially reduced the natural tropism of the virus (229).

1.7. Agents that increase AAV transduction

As mentioned above, AAV infects different cell types to varying degrees. Often times, higher expression is desired than what is observed following administration of the virus alone. To this end, several agents have been examined for their ability to increase AAV transduction.

1.7.1. Proteasome inhibitors

Two separate classes of proteasome inhibitors, tripeptidyl aldehydes and anthracycline derivatives, can synergistically augment AAV transduction in human airway epithelia, suggesting these different classes of inhibitors modulate different proteasome functions that mediate AAV transduction (230). In polarized human airway epithelia, the proteasome inhibitor LLnL was able to increase transgene expression after apical infection with AAV2 or AAV5 vectors. Interestingly, basal infection was unaffected by the presence of the proteasome inhibitor. Both wild type or self-complimentary vectors provided similar expression levels, suggesting that second-strand synthesis is not a rate-limiting step of AAV transduction following apical infection of polarized human airway epithelia (231).

Proteasome inhibitors also increase transgene expression in human intestinal epithelial cells *in vitro* up to 400-fold (232). A dual therapeutic use of proteasome inhibitors in human cystic fibrosis airway epithelia infected with AAV has been observed. Not only was there an increase in CFTR transgene expression, but there was also a simultaneous inhibition of ENaC currents that was independent of vector administration (233).

1.7.2. DNA damaging agents

The DNA damaging minor groove binding drugs Hoechst 33258 and Hoechst 33342 increased AAV transgene expression in HEK293 and COS-1 cells. The effect was independent of the presence of AAV particles, as plasmid transfected cells demonstrated similar effects. However, the presence of viral ITRs in the plasmid construct was necessary, as their removal completely abrogated the effects of these agents (234). UV-irradiation can also increase AAV transduction in human fibroblast-like synoviocytes, articular chondrocytes, and mesenchymal stem cells (235-237). Genotoxic agents such as etoposide and γ-irradiation were able to increase AAV-mediated transduction of hepatocellular carcinoma cells *in vitro*, although only γ-irradiation increased tumor transduction *in vivo* (238). γ-rays were also able to increase transduction of a human maxillary sinus cancer cell line by increasing the rate of second-strand synthesis (239). DNA damaging agents such as γ-irradiation, UV irradiation, cisplatinum, and tritiated thymidine significantly increased AAV2 transduction in primary human foreskin fibroblasts (240). The DNA synthesis inhibitors aphidicolin and hydroxyurea and topoisomerase inhibitors such as etoposide or camptothecin also increased AAV transduction dramatically in primary human fibroblasts (241).

Ataxia telangiectasia is caused by a genetic defect in the *ATM* gene and results in increased sensitivity of cells to DNA damaging agents like γ-irradiation. Fibroblasts isolated from patients with ATM deficiency had dramatically increased AAV transduction compared to normal fibroblasts. The mechanism appeared to be independent of endocytosis or nuclear trafficking. Instead, ATM deficient cells demonstrated increased circular and integrated forms of the viral genome (242).

1.7.3. Adenovirus enhances AAV transduction

Adenovirus infection increases AAV transduction dramatically by increasing second strand synthesis (180, 181). More specifically, adenoviral proteins E4 ORF6 and E1b 55 kDa protein have been demonstrated to be responsible for mediating this effect in a p53-independent manner (243). The presence of the 34 kDa protein from E4 ORF6 appears to lead to a degradation of cyclin A, which disrupts cell cycle progression and leads to increased AAV transduction (244). Others have suggested that although adenovirus can increase the amount of transgene expression initially, over time expression in the absence of adenovirus will be equal to the adenovirus treated group, indicating a delay in the double strand conversion of AAV genomes rather than an absolute requirement for adenovirus (141). Although adenovirus protein E4 ORF6 and UV irradiation both appear to increase AAV transduction, they do so by different mechanisms. UV irradiation increases the circular form of the viral genome, while E4 ORF6 increases replication form intermediates (245).

1.7.4. Tyrosine phosphatase activation

Phosphorylated forms of the cellular protein FKBP52 have been demonstrated to inhibit viral second-strand synthesis both in HeLa cells *in vitro* and in murine hepatocytes *in vivo* (246, 247). The cellular protein T cell protein tyrosine phosphatase (TC-PTP) can dephosphorylate FKBP52 and subsequently leads to increases in viral second-strand synthesis and in AAV transduction in both human cell lines and primary murine hematopoetic cells from TC-PTP transgenic mice *in vitro* (248, 249). Both FKBP52 knockout mice and TC-PTP transgenic mice demonstrate increased hepatic transduction following intravenous administration of AAV. With this in mind, it has been possible to increase single-stranded AAV transgene expression both *in vitro* and *in vivo* by co-infecting a self-complementary AAV vector encoding TC-PTP by increasing viral second-strand synthesis (250).

1.7.5. Tyrosine kinase inhibitors

Tyrphostin, an epidermal growth factor receptor tyrosine kinase inhibitor, was able to increase AAV transduction in several cell lines *in vitro*, acting by decreasing phosphorylation of single-stranded D sequence-binding protein (ssD-BP) and leading to increased transgene expression (251). More specifically, in a transformed human lung epithelial cell line, tyrphostin appeared to act by upregulating the stress response protein kinase pathways p38 and JNK, resulting in increased transcription from linear vector DNA templates (252).

1.7.6. AAV/vector complexes

Complexing AAV vectors encoding the human insulin gene with calcium phosphate was demonstrated to enhance insulin secretion and blood glucose control in a streptozocin-induced diabetes model after injection into the liver parenchyma (253). Similarly, calcium phosphate also increased AAV transduction of human airway epithelia *in vitro* and in mouse lungs *in vivo* (254). Conjugation of AAV to polymer matrix microparticles was also able to increase AAV transduction both *in vitro* and *in vivo* (255). The addition of the polycation protamine sulfate increased AAV transduction of the hepatocyte cell line HepG2, apparently by conferring a positive charge on the viral particle, thereby leading to more adsorption of the virus on the negatively charged cells (256). Liposomes were also able to increase AAV transduction *in vitro* in a human glioma cell line (257).

1.7.7. Oxidative Stress

Oxidative stress, whether in the form of chronic ethanol exposure or ischemia-reperfusion injury, increased AAV-mediated trangene expression in rat liver in an NFκB-dependent fashion (258). Reactive oxygen species can also increase AAV transduction. Hydroxide radicals appear to modulate tyrosine phosphatase pathways involved in AAV tranduction in HeLa cells, as

demonstrated by the ability of sodium orthovanadate to prevent increased transduction in the presence of hydrogen peroxide (259). Interestingly, the antioxidant molecule and anti-HIV drug pyrrolidinedithiocarbamate (PDTC) synergistically increased the augmentation of AAV transduction in UV-irradiated or H_2O_2 treated HeLa cells (260).

1.7.8. Other agents that increase AAV transduction

Several other agents can increase AAV transduction. Co-infusion of the AAV2 with mannitol, combined with ultraslow micro-perfusion, resulted in up to a 300% increase in transduction in the rat brain (261). Perfluorochemical liquid enhances AAV2-mediated transgene expression in rodent lungs up to 26-fold (262). Hyaluronidase, an enzyme that dissociates the extracellular matrix, enhances AAV vector diffusion and subsequent transduction in rat muscle (263). CpG oligodeoxynucleotides are able to increase AAV transduction in primary B cell chronic lymphoid leukemia cells independent of the use of single-stranded or self-complementary vectors (264).

1.8. Statement of the problem

Rheumatoid arthritis and juvenile rheumatoid arthritis (JRA) are among the leading causes of disability in the developed world, affecting nearly 1% of the population. There remains no cure for these diseases and current treatments are only modestly effective at slowing disease progression and providing symptomatic relief. In addition, current treatments are often delivered systemically and require frequent administration. They are also associated with significant side effects. Local treatments often have fewer side effects but also require frequent administration. Gene transfer has the potential to overcome these obstacles.

Utilizing gene transfer approaches for the treatment of RA is particularly appealing because the joint is readily accessible by intra-articular injection. One attractive vector is the

single-stranded recombinant adeno-associated virus, a small, non-enveloped DNA parvovirus. It has been shown to deliver transgenes to a wide variety of tissues, has low immunogenicity, and is associated with long-term transgene expression. In our studies, the fibroblast-like synoviocytes are being targeted for AAV-mediated transgene delivery because of the prominent role they play in cartilage and bone destruction in RA. Unfortunately, while human FLS are permissive for AAV infection, transgene expression is relatively low, potentially limiting its use as a viable treatment for RA. Several agents have been found to increase transgene expression from AAV vectors and among them are LPS and proteasome inhibitors. For example, the inflammation associated with LPS administration increased AAV-mediated transgene expression in both a rat model of arthritis and in the corneal endothelium. The specific effect of inflammation and proteasome inhibition on AAV-mediated transgene expression in FLS has not been examined and is the focus of this dissertation.

We hypothesized that both the inflammatory mediators present in the joint in rheumatoid arthritis and proteasome inhibition would increase AAV-mediated transgene expression fibroblast-like synoviocytes. Our results demonstrate that both inflammatory cytokines and proteasome inhibitors dramatically increase AAV-mediated transgene expression in FLS. Remarkably, these agents were also able to regulate transgene expression from the vector. Clinical trials using one class of proteasome inhibitors have been successful in the treatment of multiple myeloma (265, 266). Therefore, it may be possible that they could be used in conjunction with AAV vectors in the treatment of RA. However, proteasome inhibition can be associated with undesirable side effects. More promising is the finding that inflammatory cytokines that are present in the joint could also regulate transgene expression in FLS. Since the main site of inflammation in RA is the joint, this would make local administration of virus

particularly appealing. This is important because the local inflammatory environment of the joint space may actually promote transgene expression. It has the potential to dramatically affect the course of disease while at the same time minimizing the risk of deleterious side effects often associated with systemic administration of viral gene transfer vectors. It also suggests an ability of the vector to auto-regulate transgene expression, with expression being turned on during active disease, and then turned off once inflammation is brought under control.

2. Inflammatory cytokines regulate transgene expression in fibroblast-like synoviocytes infected with adeno-associated virus

2.1. Abstract

2.1.1. Objective

An ideal gene transfer vector for chronic inflammatory diseases such as rheumatoid arthritis (RA) would provide local transgene expression only when the disease is active. To determine whether adeno-associated virus (AAV) possesses this ability, the effects of inflammatory cytokines on transgene expression were evaluated in human RA fibroblast-like synoviocytes (FLS).

2.1.2. Methods

Human fibroblast-like synoviocytes (FLS) were infected with AAV in the presence or absence of inflammatory cytokines or synovial fluid obtained from patients with RA. Transgene expression was monitored by either ELISA or flow cytometry. Transgene mRNA was measured by quantitative RT-PCR.

2.1.3. Results

Inflammatory cytokines increased transgene expression in FLS by 10 to 1,000 fold. Synovial fluid from patients with RA, but not fluid from patients without arthritis, was also able to increase expression in synoviocytes. Protein expression correlated with transgene mRNA

levels. The enhanced expression required the continued presence of cytokines as, upon removal, transgene expression returned to baseline. Expression could be repeatedly re-induced by re-exposure to cytokines. The effect was not promoter specific and was demonstrated to be PI3K and NFκB-dependent. However, these two effects were independent of one another.

2.1.4. Conclusion

These results suggest that expression of a therapeutic transgene can be controlled by the presence of inflammation following AAV gene transfer, making it an attractive vector for chronic inflammatory diseases such as RA.

2.2. Introduction

Rheumatoid arthritis (RA) is a common inflammatory disorder, with a prevalence of approximately 0.5-1.0% in the adult population. It can cause significant pathology and functional impairment in affected individuals (4, 6). The most successful treatment strategies for RA to date are aimed at neutralizing the effects of pro-inflammatory cytokines such as TNFα and IL-1β (267, 268). However, based on criteria developed by the American College of Rheumatology (ACR), less than half of patients show improvement of at least 50% in their ACR scores (12). Gene transfer strategies have the potential to overcome some of these limitations and studies looking at the efficacy of viral and non-viral vectors for transfer of therapeutic molecules have been extensively studied in animal models of arthritis (269, 270). One approach that has been suggested for the treatment of RA is the local over-expression of anti-inflammatory molecules in the joint itself, which could potentially avoid side effects associated with systemic administration. Because the joint is readily accessible by intra-articular injection, gene transfer may be a feasible option in the treatment of RA. Gene transfer using adenoviral vectors has been shown to be effective in animal models of arthritis (14, 84, 92, 271).

One of the more attractive vectors for gene transfer is adeno-associated virus (AAV). AAV is a small, non-enveloped single stranded DNA parvovirus that causes no known human pathology. AAV has low immunogenicity and mediates long-term gene expression, which are essential if gene transfer is to become a viable treatment option for chronic inflammatory diseases such as RA. Several serotypes have been identified in primates, with AAV2 being the prototype for most gene transfer studies (50). AAV vectors have been designed that are able to package double stranded viral genomes, bypassing a rate-limiting step of viral transduction (second-strand synthesis) and allowing rapid and highly efficient transduction in several cell types both *in vitro* and *in vivo* (52).

TNFα, IL-1β, and IL-6 play a prominent role in the pathogenesis of RA (7, 9, 10). TNFα and IL-1β both induce large increases in IL-6 production from RA fibroblast-like synoviocytes (FLS) (272). However, because RA FLS only express the gp130 subunit of the IL-6 receptor, soluble IL-6 receptor must be supplied for trans-signaling to occur (273). Since any gene transfer vector targeting inflamed synovium might be influenced by pro-inflammatory cytokines present in the joint, the present study examined the effects of IL-1β, TNFα, and IL-6 on transgene expression in human RA FLS infected with AAV.

2.3. Materials and Methods

2.3.1. AAV production

Single stranded (ss) and double stranded (ds) AAV vectors encoding the cDNA of the soluble TNF receptor type II (p75)-human Fcγ1 fusion protein (sTNFR-Ig), human IL-10, or enhanced green fluorescent protein (EGFP) were constructed and virus was produced and titered as previously described (274). Briefly, AAV serotype-2 was made by the 293/triple transfection method. Helper functions were provided by a plasmid containing the necessary adenoviral

genes. Virus was purified by heparin affinity chromatography and titered by DNA slot blot analysis using a transgene specific biotinylated probe. Transgene expression from the vectors was driven by either the CMV promoter/CMV enhancer or the chicken beta actin promoter/CMV enhancer, as indicated. In order for efficient packaging of sTNFR-Ig into dsAAV it was necessary to truncate the C-terminal 300bp of the Fc region. The molecule was still able to dimerize (Figure 2) and was able to neutralize TNFα similar to the full-length molecule (Figure 3). The majority of both the full length and truncated molecules were present as dimers. A small amount of the truncated molecule was also present in monomer form. Supernatants from COS7 transfected cells expressing full-length sTNFRII-Ig fusion protein were able to completely inhibit TNFα mediated apoptosis to a dilution of 1:256. Supernatants from COS7 cells expressing the truncated molecule were able to complete neutralize TNFα activity to a dilution of 1:128, indicating that while this molecule may not be quite as efficient at neutralizing TNFα as the full length molecule, it retains biologic activity.

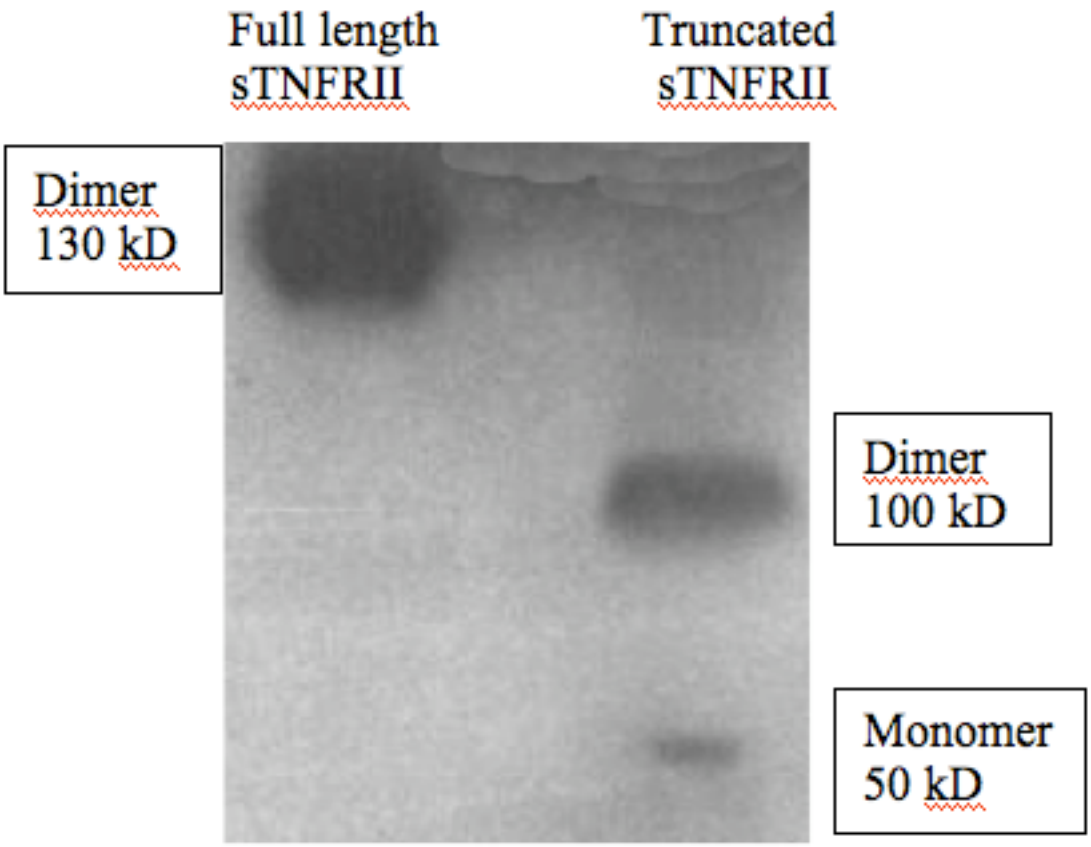


Figure 2: Dimerization of full length and truncated sTNFRII-Ig fusion protein.

Supernatants from COS7 cells tranfected with plasmids expressing either full length or truncated sTNFRII-Ig fusion protein were resolved on a 10-20% Tris-glycine gel under non-reducing conditions. Western blot was performed using a biotinylated antibody directed against sTNFRII (0.3ug/ml). Streptavidin-Horse Radish Peroxidase conjugate (Zymed) was added at a 1:2500 dilution and the blot was subsequently developed using the Pierce Supersignal West Femto Maximum Sensitivity Substrate according to the manufacturer's instructions.

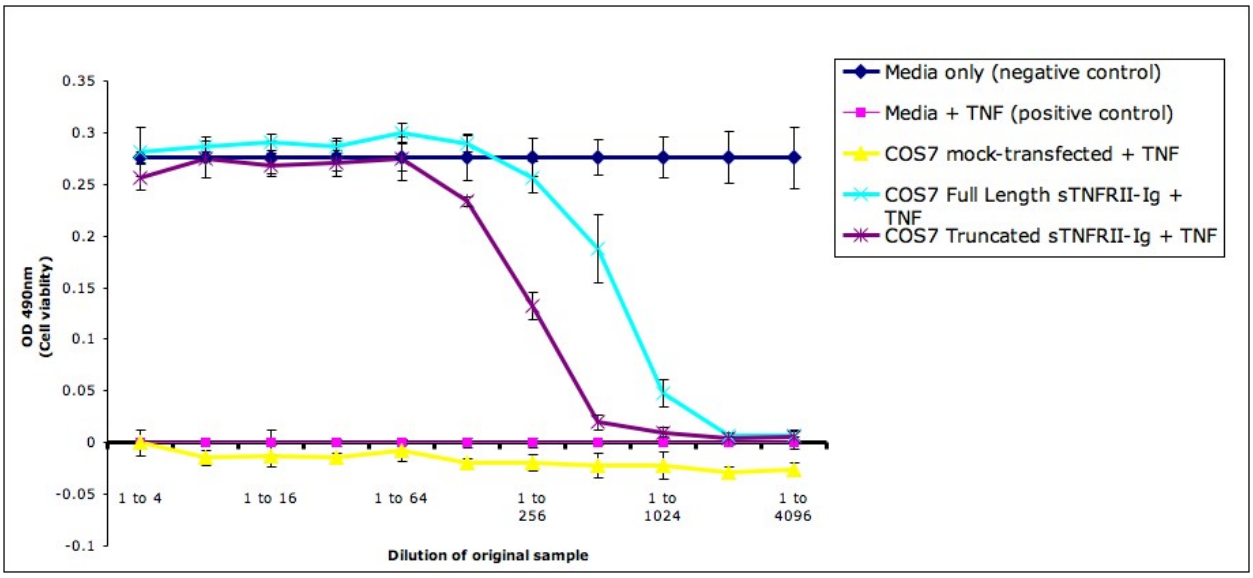


Figure 3: Ability of full length and truncated sTNFRII-Ig fusion proteins to prevent TNFα-mediated apoptosis of mouse L929 cells.

Increasing dilutions of supernatants from COS7 cells transfected with plasmids expressing either full length or truncated sTNFRII-Ig fusion protein were used to monitor their ability to prevent apoptosis of L929 cells through neutralization of TNFα.

2.3.2. Cell culture and AAV infection

Several human RA FLS primary cell lines were isolated from RA patients. Human RA synovial tissues, obtained during joint replacement surgery, were finely chopped, washed with sterile PBS, resuspended in 4mg/ml of collagenase (Worthington Biochemical Corporation, NJ) and incubated in a 37˚C CO_2 humidified incubator overnight. After washing with PBS, dissociated cells were resuspended in RPMI supplemented with 10% fetal calf serum and adherent cells were passaged in culture for 2-4 weeks. Adherent cells lines were shown to have the phenotype of fibroblast-like synoviocytes ($CD90^+$, $CD14^-$) by flow cytometry (275).

Hs69 dermal fibroblasts, COS7 monkey kidney fibroblasts, 293 cells, HeLa cells, Saos-2 osteosarcoma cells, and MRC5 lung fibroblasts were obtained from the American Type Culture Collection (ATCC). Indicated cells were plated at a density of $5x10^4$ cells in 24-well plates

(Falcon) in the presence or absence of TNFα (10ng/ml) (R&D Systems, Minneapolis, MN), IL-1β (1ng/ml) (R&D Systems, Minneapolis, MN), and/or soluble IL-6 receptor (50ng/ml) (Research Diagnostics, Inc., Flanders, NJ). The cells were then infected with $1x10^4$ particles/cell of AAV for 6 hours in a 300μl volume, washed twice with PBS, and incubated with fresh media containing the indicated cytokines. Supernatants were collected at the indicated time points and transgene expression was monitored by ELISA (for sTNFR-Ig and IL-10) or flow cytometry (for EGFP) and compared to uninfected controls. Testing of synovial fluid samples was performed as above with the following exceptions. $1x10^4$ cells were seeded in a 96 well plate and cells were incubated with 1:4 dilutions of synovial fluids obtained either from patients diagnosed with juvenile rheumatoid arthritis or from patients who underwent surgical procedures for an orthopedic injury. The cells were infected in a 100μl volume.

2.3.3. Flow cytometry

Cells were trypsinized, resuspended in PBS, pelleted, and resuspended in 0.5 ml buffer (0.2% BSA, 0.012% sodium azide in PBS). Samples were analyzed using the FACSAria flow cytometer (BD Biosciences, San Jose, CA) and FloJo software (Tree Star, San Mateo, CA).

2.3.4. ELISA

ELISA analysis was performed using anti-human IL-10 or anti-human TNF receptor type II (CD120b) monoclonal and biotinylated antibodies (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

2.3.5. Quantitative PCR

RNA was isolated using the RNAqueous Kit (Ambion, Austin, TX). RNA (500ng) was used as a template for Superscript RT to synthesize cDNA (Invitrogen, Carlsbad, CA). Two microliters of cDNA was used as template for quantitative PCR using the MxP3000 real time

PCR system (Stratagene, La Jolla, CA). Transgene copy number was normalized to 18S rRNA copy number. Primers sequences used were: sTNFR-Ig 5'GAATGCAAGCATGGATGCAGTCTG3' and 5'CGGTGGGCATGTGTGAGTTTTG3'; 18S rRNA 5'CGCAAATTACCCACTCCCGAC3' and 5'GCCCGCTCCCAAGATCCAACTA3' (Integrated DNA Technologies, Coralville, IA).

2.3.6. PI3K and NFκB inhibition

PI3K inhibition was achieved with 1μM wortmannin (Biosource International, Camarillo, CA). Cells were maintained in the presence of wortmannin for the entire length of the experiments, unless otherwise indicated.

In order to inhibit NFκB, Hs69 dermal fibroblasts were infected with a retrovirus encoding a super IκB repressor and containing a puromycin resistance gene (276). Following one week of selection in 2μg/ml puromycin, cells were expanded and tested for NFκb activation. Cells were transfected with a plasmid with the luciferase reporter gene under control of an NFκB responsive element (kindly provided by Dr. Jay Kolls) using the Fugene 6 transfection reagent as described by the manufacturer (Roche Diagnostics, Basel, Switzerland). 48 hours later the cells were assayed for luciferase activity using the Promega Bright-Glo Luciferase Assay System according to the manufacturer's instructions (Promega, Madison, WI). Luciferase units were measured using a Packard microplate scintillation and luminescence counter.

2.3.7. Immunohistochemistry

Cells were seeded on coverslips in tissue culture dishes. At the time of analysis cells were washed five times with PBS and then fixed with 4% paraformaldehyde in PBS for 20 minutes in the dark. Cells were washed five times with ice cold PBS and incubated with 0.1% Triton X-100 in PBS and incubated for 20 minutes in the dark. Cells were washed again five

times with ice cold PBS and then blocked for 2 hours with 5% goat serum in 0.15% glycine, 1% BSA in PBS. Cells were washed five times with ice cold PBS and the viral particles were stained with a mouse monoclonal anti-AAV antibody (clone A20) that detects only intact capsids (ARP American Research Products, Belmont, MA) at a 1:80 dilution. Cells were incubated in the dark for 1 hour at room temperature and then washed five times with ice cold PBS. For detection, a secondary goat anti-mouse IgG antibody conjugated to Alexa Fluor 568 (Molecular Probes, Invitrogen Corporation, Carlsbad, CA) was added at a 1:200 dilution in 0.15% glycine, 1% BSA in PBS and the cells were incubated in the dark for 1 hour at room temperature. Cells were washed five times with ice cold PBS and the nuclei were counterstained with a 1:500 dilution of the nuclear dye DRAQ5 (Biostatus, England) in PBS for 30 minutes. Cells were washed five times with ice cold PBS and the coverslips were placed onto slides containing a drop of gelvatol mounting medium. The cells were examined using an Olympus Fluoview 500 Confocal Microscope.

2.3.8. Cell transfection

293 cells were electroporated using a Biorad Gene Pulser. 800µl of a 293 cell suspension ($2.6x10^6$ cells/ml in RPMI) was placed in a 0.4cm cuvette. 10µg of plasmid DNA was added and the cuvette was incubated for 20 minutes at room temperature. The cells were then electorporated at 250 Volts and 960 µF capacitance. The cells were then transferred to 10ml of prewarmed RPMI and plated in a 35mm cell culture dish. 48hrs later the antibiotic G418 was added to a final concentration of 800µg/ml. The pAAV IL-10 plasmid was the same plasmid used to make the dsAAV (IL-10) virus as described above. pEGFP was obtained from Clonetech.

2.4. Results

2.4.1. Inflammatory Cytokines Increase AAV-mediated Transgene Expression in Human RA FLS

To determine the potential effects of inflammatory cytokines on AAV mediated transgene expression in synoviocytes, human RA FLS were infected with either ssAAV or dsAAV encoding EGFP in the presence of IL-1β, TNFα and/or sIL-6R. The addition of inflammatory cytokines consistently increased both the number of cells expressing EGFP and the intensity of expression. Various concentrations and time courses of exposure to cytokines were tested. We found that 10ng/ml TNFα, 1ng/ml IL-1β, and 50ng/ml sIL-6R gave the best results. In addition, the largest increases in transgene expression were seen after at least 5 days of cytokine exposure. Of all the possible permutations of cytokine combinations tested, IL-1β alone, or all three cytokines in combination, gave the largest increases in EGFP expression (Figure 4). Similar results were observed using a second human RA FLS cell line, and an osteoarthritis (OA) FLS cell line (Figure 5).

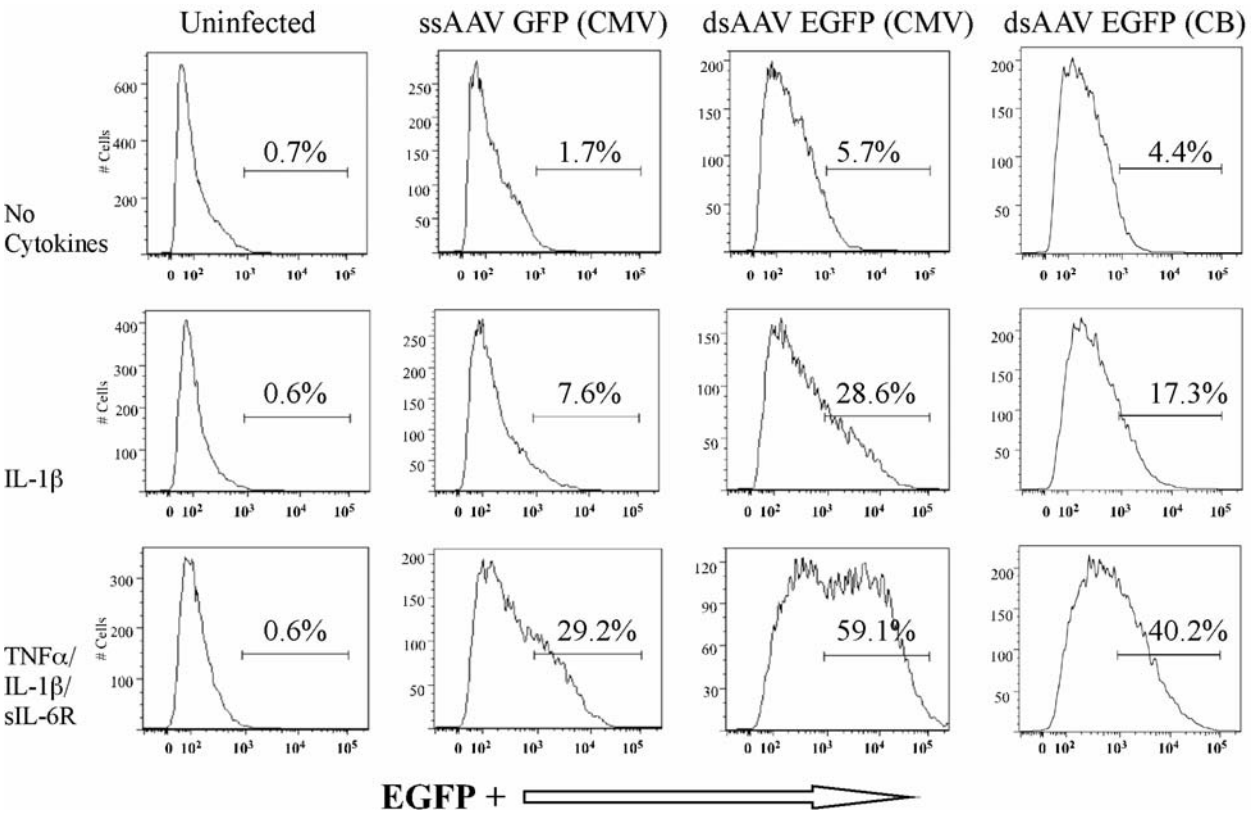


Figure 4: Inflammatory cytokines act synergistically to increase EGFP expression from RA fibroblasts infected with single stranded (ss) or double stranded (ds) AAV.

RA FLS were incubated with the indicated cytokines for 5 days. On day 3, FLS were infected with $1x10^4$ particles/cell AAV-EGFP driven by either the CMV or the chicken b-actin (CB) promoter. EGFP expression was determined by flow cytometry by comparison to uninfected cells 48 hours after infection and the data was analyzed using FloJo software. The data are representative of three independent experiments.

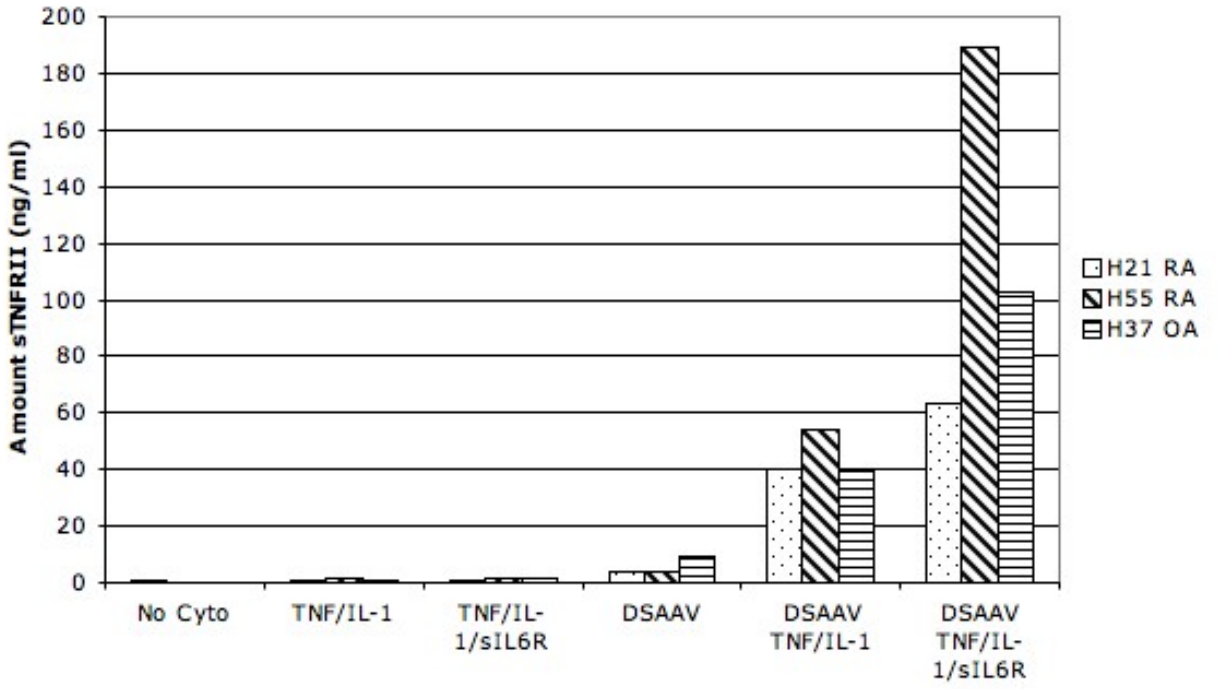


Figure 5: The effect of cytokines on dsAAV (sTNFRII) transgene expression in fibroblast-like synoviocytes isolated from both RA (H21, H55) and OA (H37) patients.

$5x10^4$ RA or OA FLS were incubated in the presence or absence of inflammatory cytokines for 72 hours prior to infection with ds AAV (sTNFRII) as described in the Materials and Methods. The indicated cytokines were added back and 48 hours after infection the supernatants were collected. Transgene levels were measured by ELISA.

The cytokine-induced enhancement of expression was not due to increased attachment or entry of virus into the cell, as similar results were obtained when the addition of cytokines to the cell cultures was performed 3 days after infection (Figure 6). To rule out any promoter-specific effects on transgene expression, dsAAV encoding EGFP with expression driven by the chicken beta actin (CB) promoter was also tested. EGFP expression from this virus also increased dramatically (Figure 4).

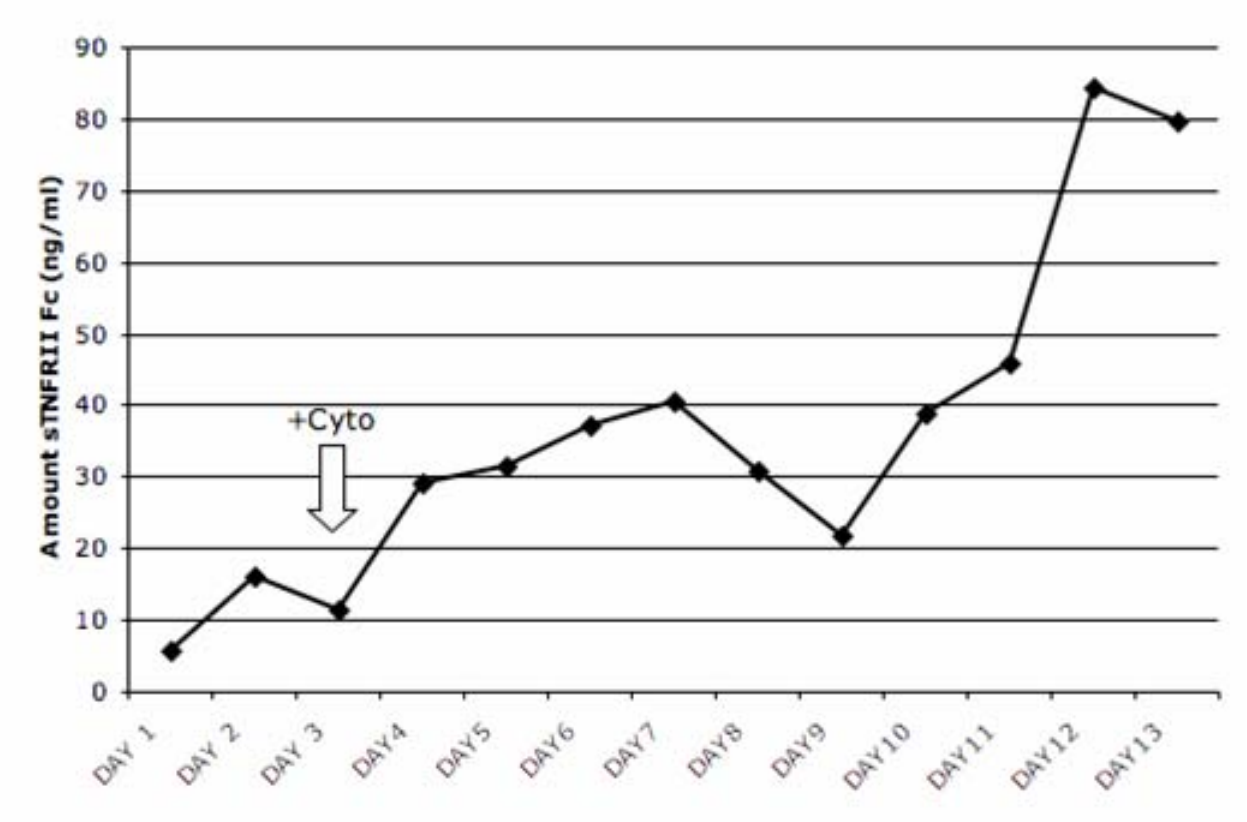


Figure 6: The effect of cytokines on ds AAV transgene expression when added three days after infection.

$5x10^4$ RA FLS were seeded in a 24 well tissue culture dish. 72 hours later the cells were infected with ds AAV (sTNFRII) as described in the Materials and Methods. Three days later inflammatory cytokines (TNFα, IL-1β, and sIL-6R) were added at the concentrations previously described. Supernatants were collected daily and transgene expression was measured by ELISA.

IL-17, another inflammatory cytokine involved in the pathogenesis of RA, was also tested and had no effects on transgene expression from rAAV vectors, either alone or in combination with any of the other cytokines tested above (Figure 7). Unless otherwise indicated, subsequent experiments were performed by incubating FLS with cytokines for 72hrs prior to infection with AAV.

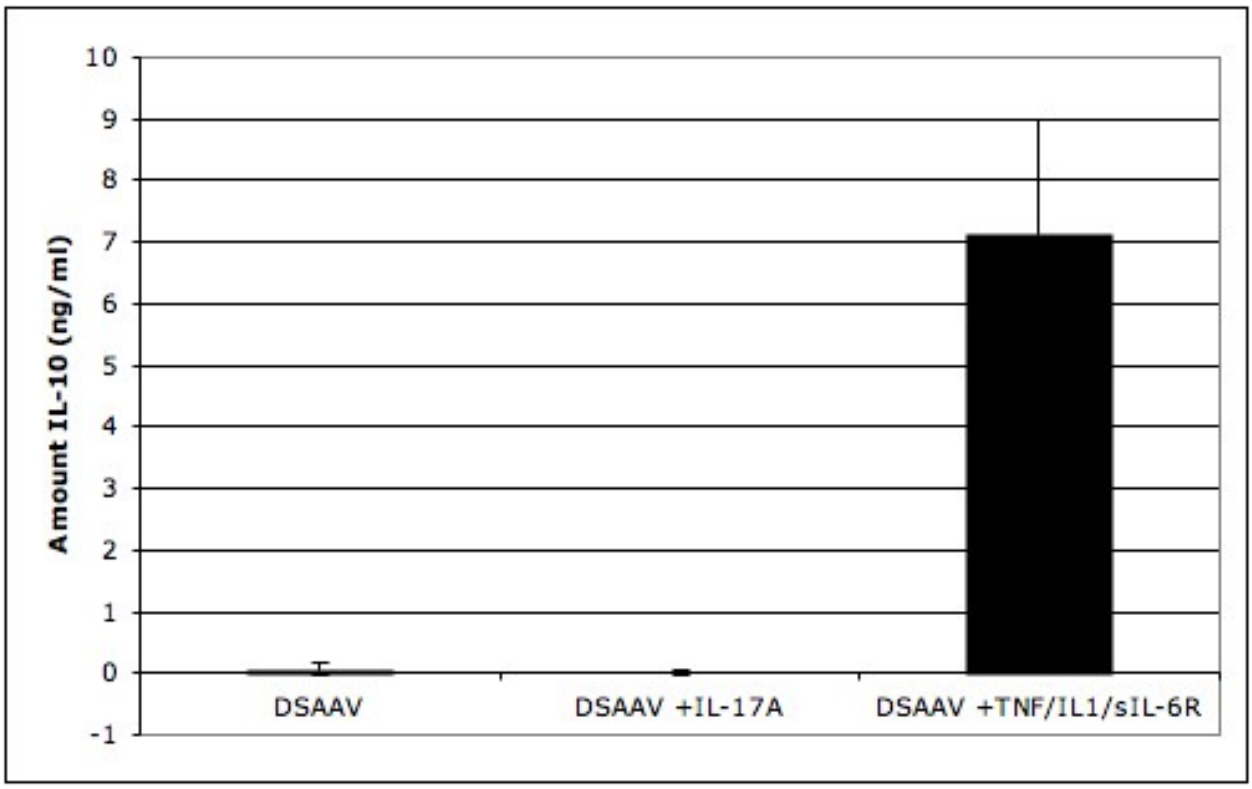


Figure 7: IL-17A has no effect on ds AAV transgene expression in human dermal fibroblasts.

$5x10^4$ dermal fibroblasts were seeded in a 24 well tissue culture dish and incubated for 72 hours in the presence of the indicated cytokines. 72 hours later cells were infected with ds AAV (IL-10) as described in the Materials and Methods. After infection, the cells were exposed to the indicated cytokines for 48 hours, at which time the supernatants were collected and transgene expression was measured by ELISA.

To determine whether similar effects could be observed using therapeutically relevant transgenes, we constructed AAV vectors encoding human IL-10 (Figure 8A). Supernatants were collected 48 hours after infection and transgene levels were measured by ELISA. Once again, inflammatory cytokines increased transgene expression in AAV-infected cells. As before, the combination of all three cytokines had the maximal effect on transgene expression, increasing expression approximately 8-fold. Similar experiments were performed using dsAAV encoding soluble TNF receptor type II (p75)-human Fcγ1 fusion protein (sTNFR-Ig) with similar results (data not shown). Although an increase in transgene expression was also observed using ssAAV (data not shown), dsAAV consistently gave a log-fold higher expression than its ssAAV

counterpart. To determine if the inflammatory environment of the joint would also be able to increase AAV transgene expression, similar experiments were performed using synovial fluid isolated either from patients diagnosed with juvenile rheumatoid arthritis or from patients who underwent a surgical procedure for orthopedic injury (Figure 8B). Synovial fluid from patients with arthritis increased AAV transgene expression 2 to 14-fold, while synovial fluid from patients without arthritis had no effect on transgene expression. The levels of TNFα (16.2 +/- 5.4 pg/ml), IL-1β (10.4 +/- 6.6 pg/ml), and especially IL-6 (2409 +/- 978 pg/ml) were higher in the inflammatory synovial fluids compared to the controls (all cytokines < 8pg/ml). In addition, the concentration of IL-10 was less than 10 pg/ml in all samples tested (data not shown), indicating that the IL-10 measured by ELISA was delivered by AAV and was not due to IL-10 present in the synovial fluids.

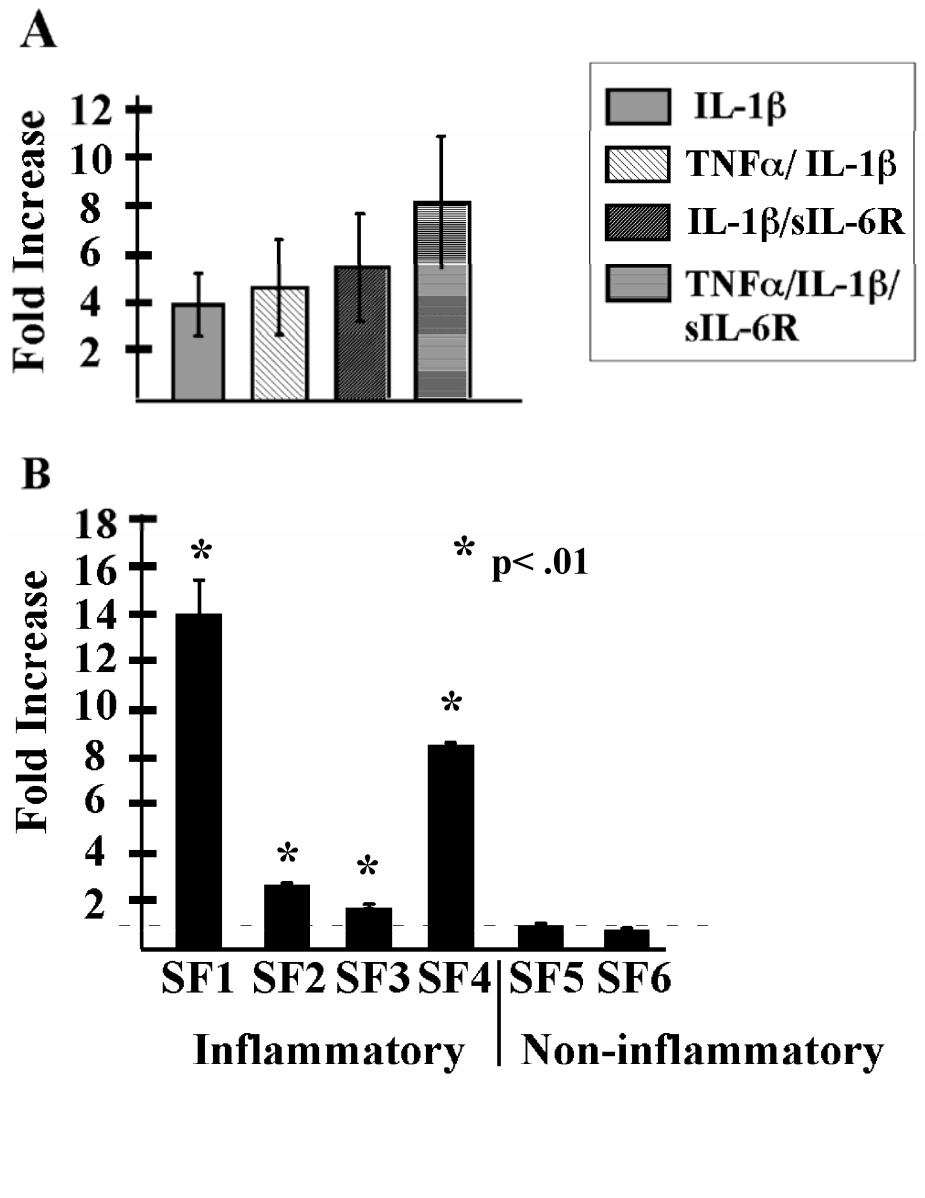


Figure 8: Inflammatory cytokines (A) and arthritic synovial fluid (B) increase human IL-10 expression from RA FLS infected with dsAAV.

RA FLS were exposed to cytokines or synovial fluid (SF) for 5 days. Synovial fluids were either from patients diagnosed with arthritis (Inflammatory) or from patients who underwent surgery for an orthopedic injury (Non-inflammatory). On day 3, the cells were infected with $1x10^4$ particles/cell AAV (encoding human IL-10) and then re-exposed to indicated cytokines or synovial fluids. Supernatants were collected 48 hours after infection and transgene levels were determined by ELISA. Fold increase in expression was obtained by comparison to FLS infected with dsAAV in the absence of any cytokines or synovial fluid. The data represent the mean and standard deviation of three replicates per group and are representative of three independent experiments (* indicates p< .01 compared to each non-inflammatory control by paired t-test).

The effect is not due to any effects the inflammatory cytokines might have on cell proliferation. Cells were counted at the conclusion of the assay and no significant differences were observed between cells treated with or without cytokines (Figure 9).

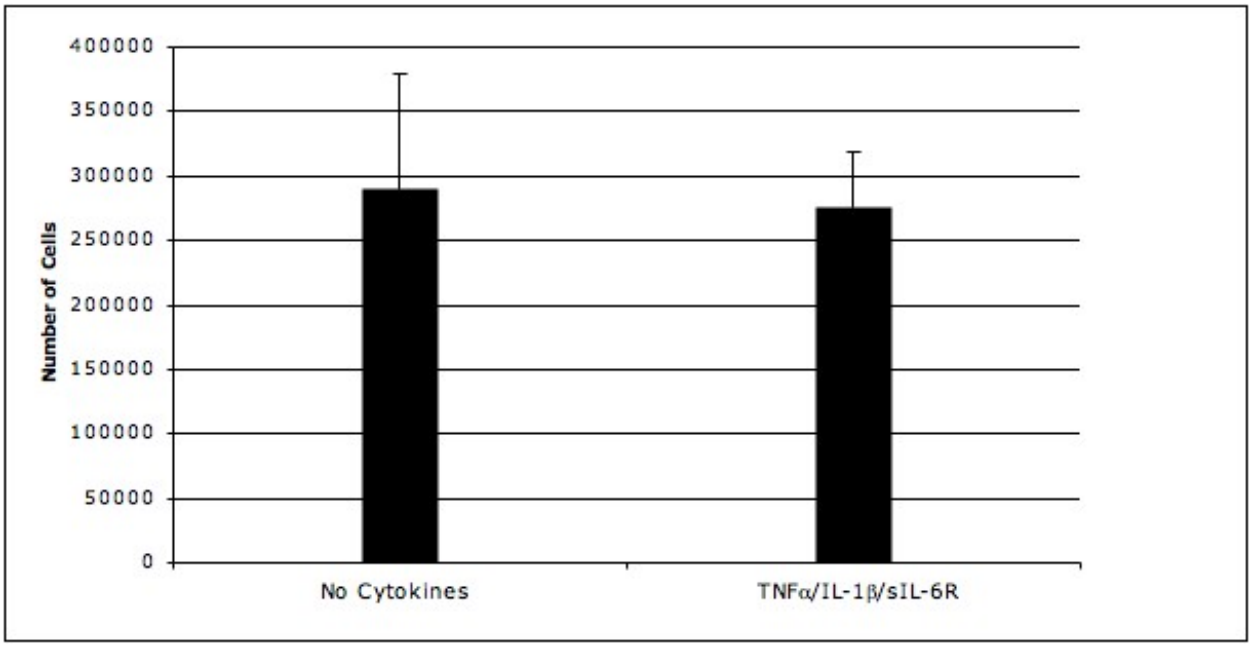


Figure 9: Inflammatory cytokines do not effect cell proliferation.

Cells were counted using a hemacytometer at the conclusion of the experiment. No significant differences were observed between groups, two of which are represented above.

Next, we examined the effects of inflammatory cytokines on mRNA levels. 48 hours after infection mRNA levels of the transgene were measured by quantitative RT-PCR and correlated with increases in protein levels (Figure 10). TNFα and IL-1β addition gave a 5-fold increase in transgene mRNA levels, while the combination of all three inflammatory cytokines gave a 40-fold increase in mRNA levels.

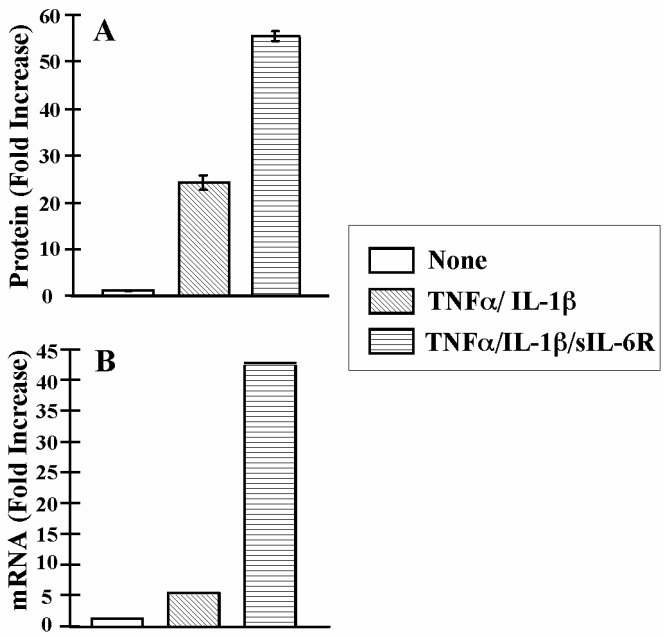


Figure 10: Transgene mRNA levels correlate with increases in protein levels following cytokine exposure.

RA FLS were incubated with the indicated cytokines for 5 days. On day 3, FLS were infected with $1x10^4$ particles/cell AAV (encoding sTNFR-Ig). (A) Supernatants were collected 48 hours after infection and transgene levels were measured by ELISA. The data represent the mean and standard deviation of three replicates per group. (B) Transgene mRNA levels were determined using quantitative PCR for sTNFR-Ig with normalization to 18S rRNA. The data are representative of three independent experiments.

2.4.2. AAV Transgene Expression Can Be Regulated By Inflammatory Cytokines

Because the inflamed joint is chronically exposed to high levels of inflammatory mediators, we sought to determine the effect on AAV transgene expression of longer-term exposure to cytokines. Exposure to any 2 cytokines led to a peak in transgene expression on day 2 after infection, followed by a steady-state expression until the conclusion of the analysis on day 12 (Figure 11A). However, when FLS were exposed to the combination of all three inflammatory cytokines, transgene expression increased after day 7 by an additional 5-10 fold. This was a consistent finding observed in three repetitions of this experiment.

Expression of the transgene was dependent on continued exposure to cytokines, as cytokine removal resulted in return of transgene expression to near basal levels within 3 days

Figure 11B). Furthermore, transgene expression could be re-induced by re-exposure to cytokines, demonstrating the ability of the cytokines to regulate transgene expression. This is important because it raises the possibility that the inflammatory environment of the joint could significantly increase the level of a therapeutic transgene delivered by AAV. However, once the inflammation is brought under control, the level of expression of the transgene would return to much lower levels. This self-regulatory mechanism is a feature that may make AAV especially suited for the treatment of inflammatory diseases like RA.

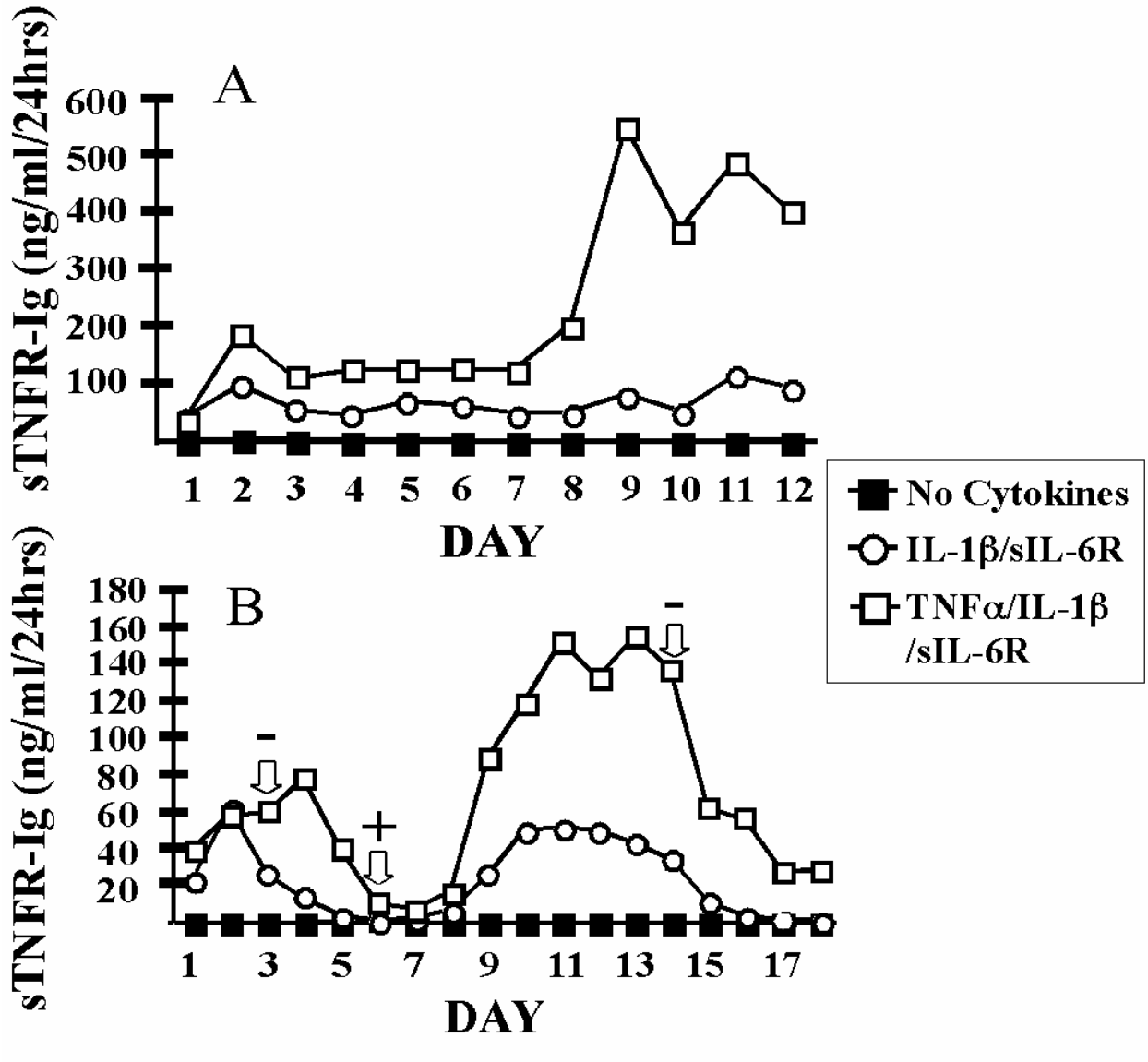


Figure 11: AAV-mediated transgene expression is regulated by inflammatory cytokines.

(A) RA FLS were infected with AAV on day 0 and exposed to cytokines on day 2. Supernatants were collected daily until day 12 and transgene expression was measured by ELISA. (B) RA FLS were exposed to cytokines for 5 days. On day 3 the cells were infected with AAV. Cytokines were then removed (-) and added (+) at indicated time points after AAV infection. Supernatants were collected every 24 hours and transgene expression was measured by ELISA. Data are representative of three independent experiments.

2.4.3. The Effect of Inflammatory Cytokines on AAV Mediated Transgene Expression is PI3K-dependent

In order to explore the mechanism by which inflammatory cytokines act to increase transgene expression, we first sought to examine the role of PI3K, an enzyme whose activation has previously been suggested to be required for efficient trafficking of AAV to the nucleus (165). FLS were infected with dsAAV-sTNFR-Ig. Three days after infection, cytokines were added with or without the PI3K inhibitor wortmannin (1μM). Wortmannin prevented increased transgene expression in response to inflammatory cytokines (Figure 12A) and did so in a dose-dependent manner (Figure 13). This suggests that the effect of the cytokines on transgene expression occurs downstream of viral entry into the cell. The effect of wortmannin was reversible, as its removal three days after infection, followed by re-exposure to cytokines on day six, led to increased transgene expression (Figure 12B).

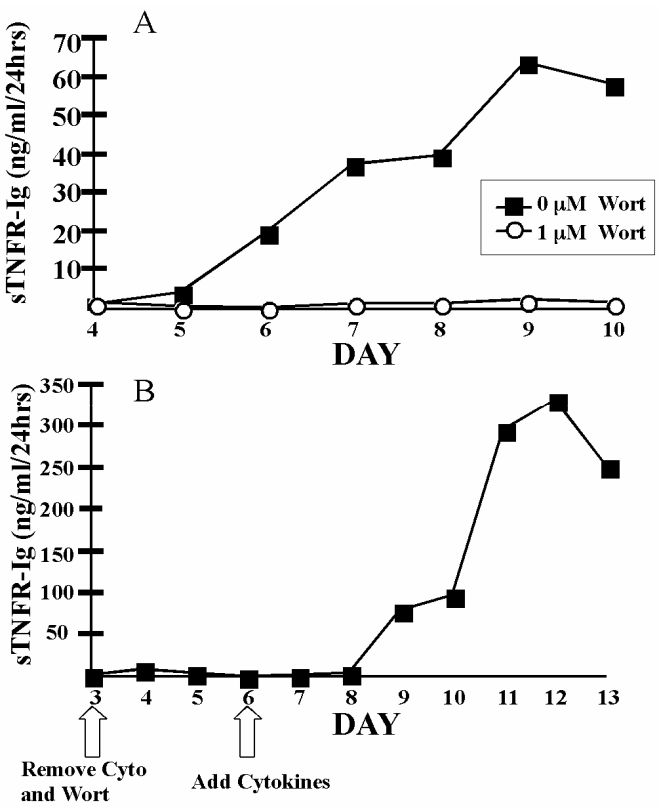


Figure 12: The effects of cytokines on AAV-mediated transgene expression are PI3K-dependent.

(A) RA FLS were infected on day 0 with $1x10^4$ particles/cell AAV (sTNFR-Ig). TNFα, IL-1β, and sIL-6R +/- wortmannin (1 μM) were added on day 3 after infection. Supernatants were collected daily and transgene expression was monitored by ELISA. (B) RA FLS were incubated with cytokines and wortmannin for 72 hrs prior to infection with AAV on day 0, and incubated in the continued presence of cytokines and wortmannin until day 3, when both were removed. On day 6 cytokines alone were added back for the remainder of the experiment. Supernatants were collected daily throughout the experiment and transgene expression was determined by ELISA. These results are representative of three separate experiments.

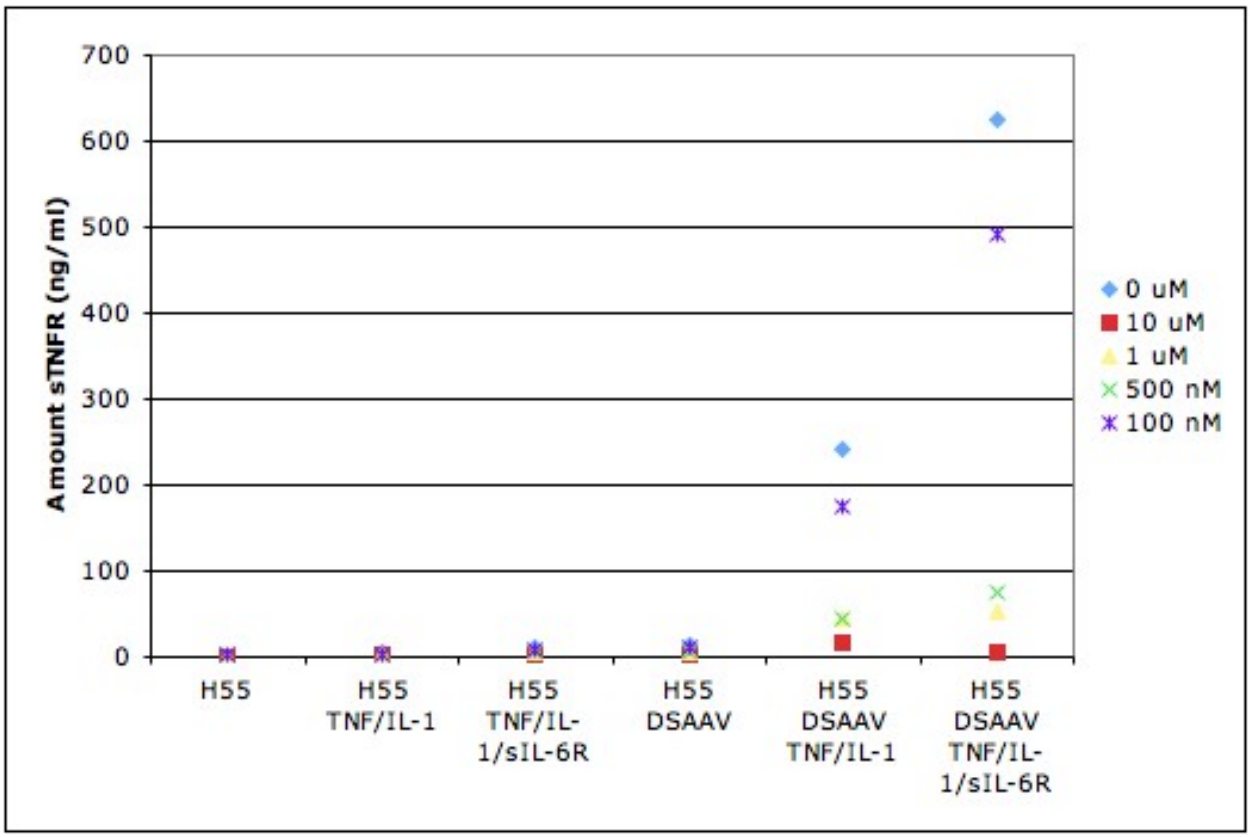


Figure 13: The effect of wortmannin on cytokine-mediated increases in dsAAV transgene expression is dose-dependent.

$5x10^4$ RA FLS (H55) were seeded in a 24 well plate in the presence of the indicated cytokines and varying concentrations of wortmannin (0 μM to 10 μM). 72 hours later the cells were infected with dsAAV (sTNFRII) as previously described. The cells were exposed to cytokines and wortmannin for an additional 48 hours and the supernatants were collected for measurement of transgene levels by ELISA.

2.4.4. The Effect of Inflammatory Cytokines on AAV Mediated Transgene Expression is NFκB-dependent

Next, we sought to determine if the transcription factor NFκB played any role in the effect of cytokines on AAV transgene expression. The AAV vectors used in our experiments, unless otherwise noted, contain the CMV promoter and enhancer to drive transcription of the transgene. There are four consensus binding sites for NFκB in the CMV promoter and enhancer regions. NFκB activity is required for efficient transcription and gene expression from

constructs containing the CMV promoter and enhancer both *in vitro* and *in vivo* (277). However, binding sites for other transcription factors are also present, including AP1, SP1, and MDBP (278). Dermal fibroblasts were infected with a retrovirus encoding a super IκB inhibitor, in which the IκBα molecule is mutated. The mutation prevents the molecule from being phosphorylated by IκB kinase and subsequently degraded, which is normally required for the translocation of NFκB to the nucleus (279). To confirm that NFκB was inhibited, the cells were transfected with a reporter plasmid containing an NFκB responsive element and encoding luciferase. 48 hours after transfection, luciferase activity was measured. The cells containing the inhibitor had approximately 10-fold lower luciferase activity, indicating that NFκB activation was impaired (Figure 14).

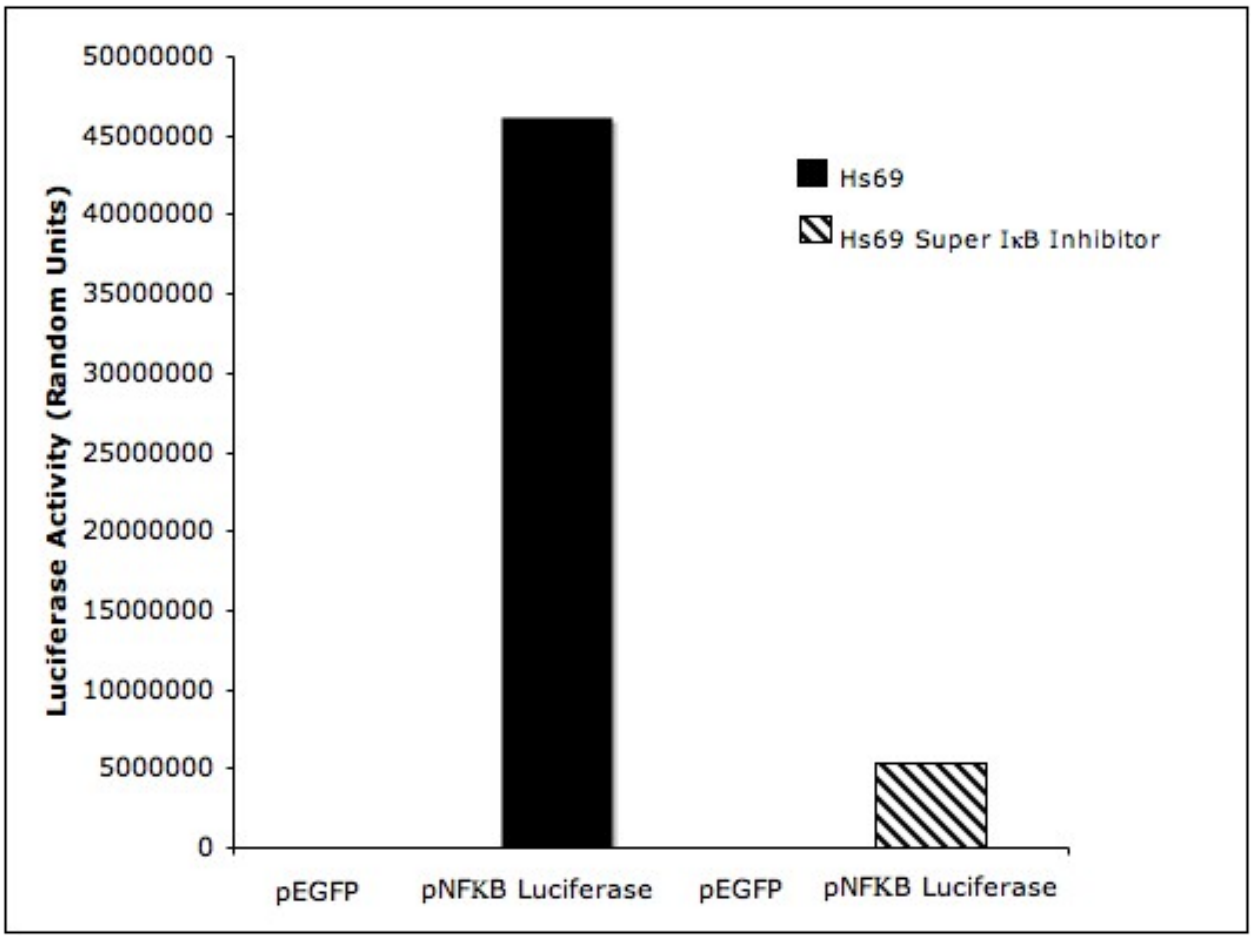


Figure 14: Inhibition of NFκB in dermal fibroblasts (Hs69) by the super IκB inhibitor.

Dermal fibroblasts expressing the super IκB inhibitor and control fibroblasts were transiently transfected with either a negative control plasmid (pEGFP) or a plasmid containing an NFκB responsive element driving luciferase expression (pNFκB Luciferase). 48 hours after transfection the cells were harvested and luciferase activity was measured using Promega's Bright-Glo Luciferase Assay System. These results are representative of two separate experiments.

After confirming that NFκB activation was inhibited, we wanted to determine if inflammatory cytokines could still increase AAV transgene expression in this cell line. The cells were infected with dsAAV in the presence or absence of inflammatory cytokines and transgene expression was measured by ELISA. The results demonstrate that inhibition of NFκB activation almost completely abolishes the increase in transgene expression normally observed in the

presence of cytokines (Figure 15). This suggests that inflammatory cytokines are able to increase transgene expression through activation of NFκB. NFκB activation could lead to increased trangene expression in one of two distinct ways. It could bind to the NFκB binding sites on the CMV promoter and enhancer and drive transcription of the transgene or NFκB could activate other genes that subsequently act to increase transgene expression via other mechanisms. Further experiments will have to be performed to determine the precise role of NFκB in this process.

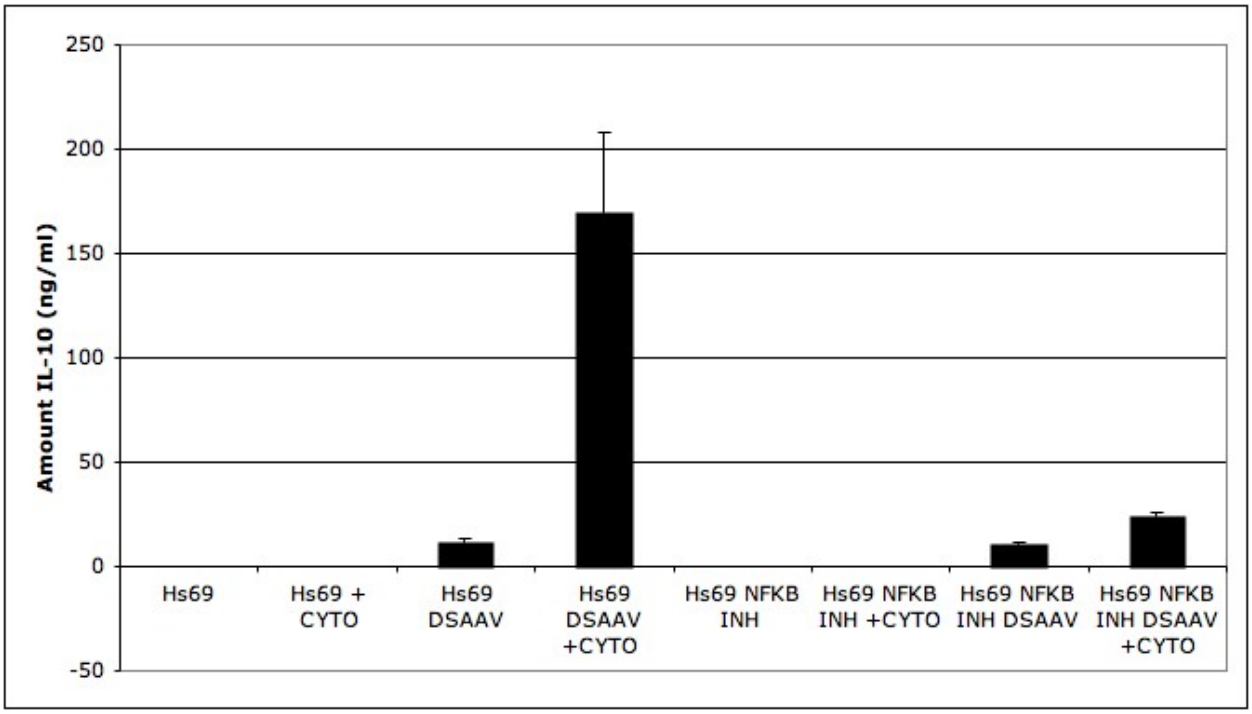


Figure 15: The effect of NFκB inhibition on the ability of inflammatory cytokines to increase AAV transgene expression in Hs69 dermal fibroblasts.

Dermal fibroblasts expressing a super IκB inhibtor (Hs69 NFκB INH) or normal dermal fibroblasts (Hs69) were exposed to cytokines (CYTO) and infected with dsAAV encoding IL-10 as described in the materials and methods. Transgene levels were measured by ELISA 48 hours after infection. These results are representative of three separate experiments.

2.4.5. NFκB and PI3K play independent roles in the cytokine-mediated increase in AAV transgene expression

The above studies demonstrate that both the PI3K pathway and activation of NFκB appear to play a role in the cytokine-mediated increases in AAV transgene expression in fibroblasts. These molecules could play independent roles in increasing AAV transgene expression in response to cytokines or they could act in concert. Activation of the PI3K pathway leads to the activation of NFκB in some cell types, while in other cells NFκB is unaffected by the activation of the PI3K pathway (280-283). To determine if the PI3K inhibitor wortmannin could also inhibit NFκB activation dermal fibroblasts were transfected with a plasmid encoding an NFκB responsive luciferase reporter gene in the presence or absence of wortmannin. 48 hours later the cells were harvested and luciferase activity was measured. The results clearly demonstrate that wortmannin clearly does not inhibit NFκB in dermal fibroblasts (Figure 16). If anything, treatment of the cells with wortmannin leads to activation of NFκB. This suggests that the PI3K pathway and NFκB mediate cytokine-mediated increases in AAV transgene expression in fibroblasts by separate mechanisms.

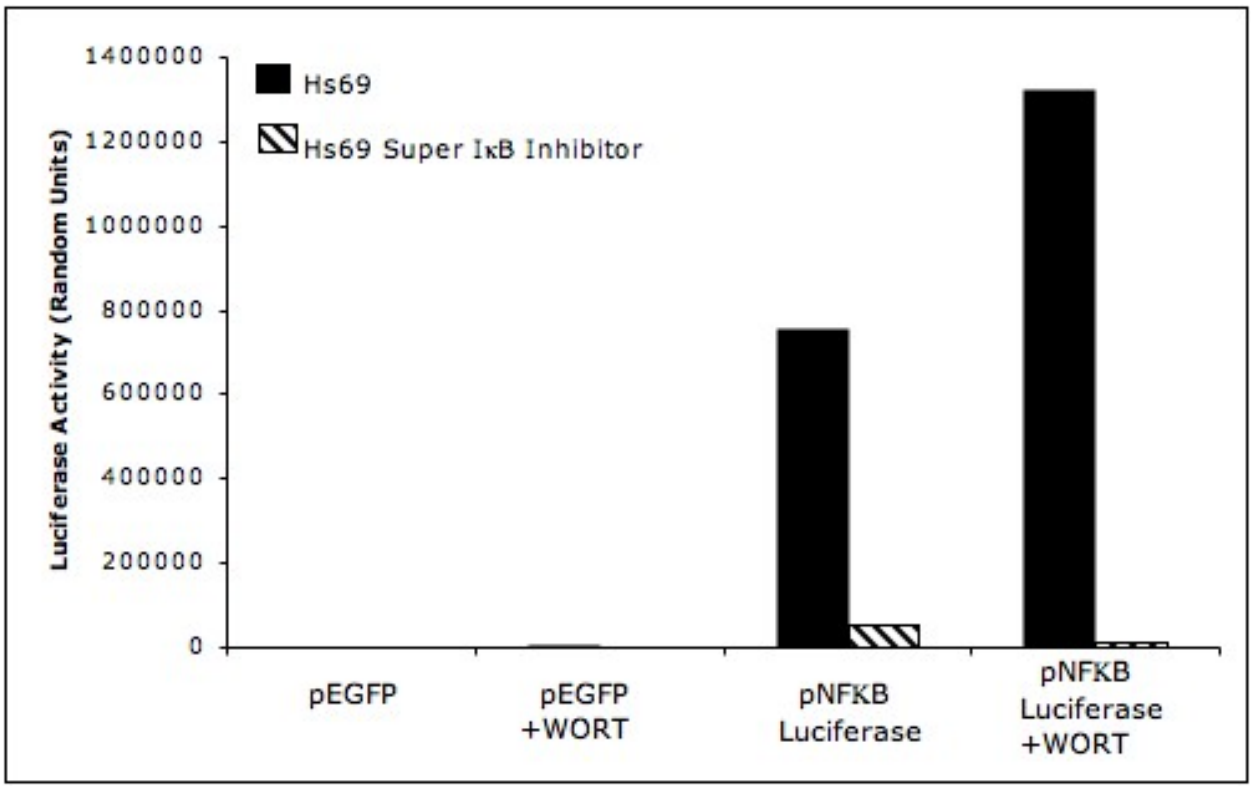


Figure 16: Wortmannin does not inhibit NFκB in dermal fibroblasts.

Dermal fibroblasts were pre-treated for 24hrs with 1μM wortmannin. The cells were then transfected with either a negative control plasmid (pEGFP) or a plasmid encoding an NFκB responsive luciferase reporter gene in the continued presence or absence wortmannin. 48 hours later the cells were harvested and luciferase activity was measured using Promega's Bright-Glo Luciferase Reporter System. Dermal fibroblasts expressing an inhibitor of NFκB were also tested as a control. These results represent one experiment.

2.4.6. Inflammatory cytokines promote uncoating of the virus

In order to further elucidate the mechanism of cytokine-mediated increases in AAV transgene expression, the intracellular localization of the virus was examined in the presence or absence of inflammatory cytokines by staining the dermal fibroblasts with an anti-AAV antibody specific for intact viral particles. Confocal microscopy revealed that while the untreated cells demonstrated staining throughout the cytoplasm, primarily in the perinuclear area, intact particles were almost completely absent in the cytokine treated cells (Figure 17). This suggests

that the cytokines promote uncoating of the virus. Whether or not this is directly related to increases in AAV transgene expression requires further exploration.

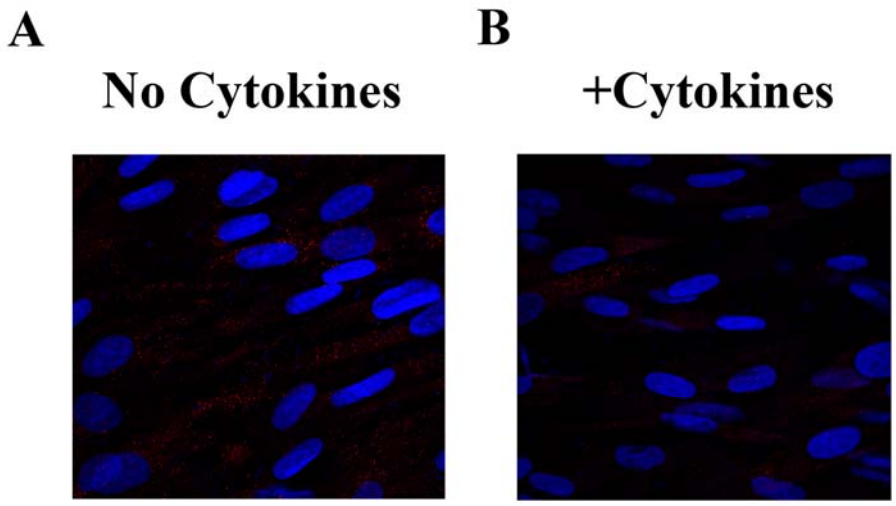


Figure 17: Inflammatory cytokines promote uncoating of AAV.

Dermal fibroblasts were incubated on coverslips in the absence (A) or presence (B) of TNFα, IL-1β, and sIL-6R for 72 hours prior to infection with dsAAV (as described in Materials and Methods). 24 hours after infection the cells were fixed and stained with a mouse monoclonal anti-AAV antibody specific for intact capsids. For detection, a secondary goat anti-mouse antibody conjugated to Alexa Fluor 568 was used (red). The cells were counterstained with the nucleic acid dye DRAQ5 (blue).

2.4.7. The effect of cytokines on AAV trangene expression is not restricted to fibroblasts

While fibroblasts are only minimally infected by recombinant AAV vectors, other cell types are more readily infectible. To determine if more permissive cell types were also susceptible to the effects of inflammatory cytokines, a panel of different cell lines was examined. Most of the cell lines tested were permissive for AAV infection, as demonstrated by the high level of transgene expression observed even in the absence of cytokines (Figure 18). These cells all demonstrated less than 2-fold induction of AAV transgene expression upon cytokine addition. Only one cell line, HEK 293 cells, appear, like human RA FLS and dermal fibroblasts, to be

minimally infected by AAV. These cells were isolated from human embryonic kidney and have an epithelial morphology, although these cells are not fully characterized. Like the fibroblasts, 293 cells also demonstrated an approximate 8-fold increase in AAV trangene expression upon exposure to cytokines. Interestingly, COS7 cells, a SV40 transformed monkey kidney fibroblast line, and MRC5 cells, a normal lung fibroblast line, appear to be permissive for AAV infection. Some fibroblasts, therefore, may not be susceptible to the effects of cytokines. Clearly different types of fibroblasts display wide variablilty in their ability to be infected with AAV. Cytokines, meanwhile, appear to improve transduction of cell types that are minimally infected by AAV.

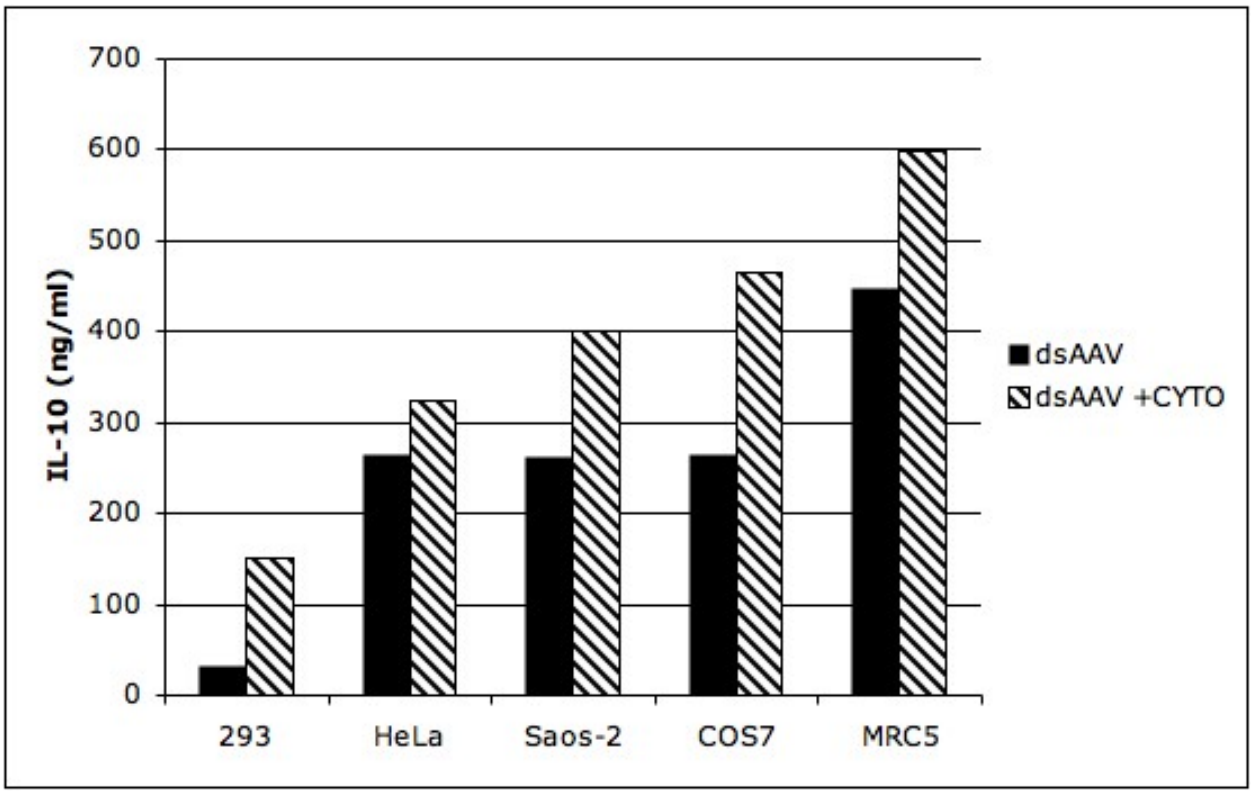


Figure 18: Effect of cytokines on AAV transduction in different cell lines.

293, HeLa, and Saos-2 cells were exposed to TNFα, IL-1β, and sIL-6R and COS7 and MRC5 cells were exposed to IL-1β for 72 hours prior to infection with dsAAV encoding human IL-10 (as described in Materials and Methods). Following infection, the cells were re-exposed to cytokines and supernatants were collected 48 hours later. Transgene expression was determined by ELISA. These results represent one experiment, which has only been repeated three times for the 293 cells.

2.4.8. Inflammatory cytokines increase gene expression from stably transfected cells

To provide more insight into the mechanism of inflammatory cytokine mediated increases in AAV transgene expression, we sought to determine if viral infection was a requirement for this phenomenon. 293 cells were stably transfected with the plasmid used to make the virus, which contains the transgene with the CMV promoter/enhancer flanked by the viral ITRs. Cytokines were able to increase gene expression up to 10-fold (Figure 19), similar to what was observed in AAV-infected cells (see above).

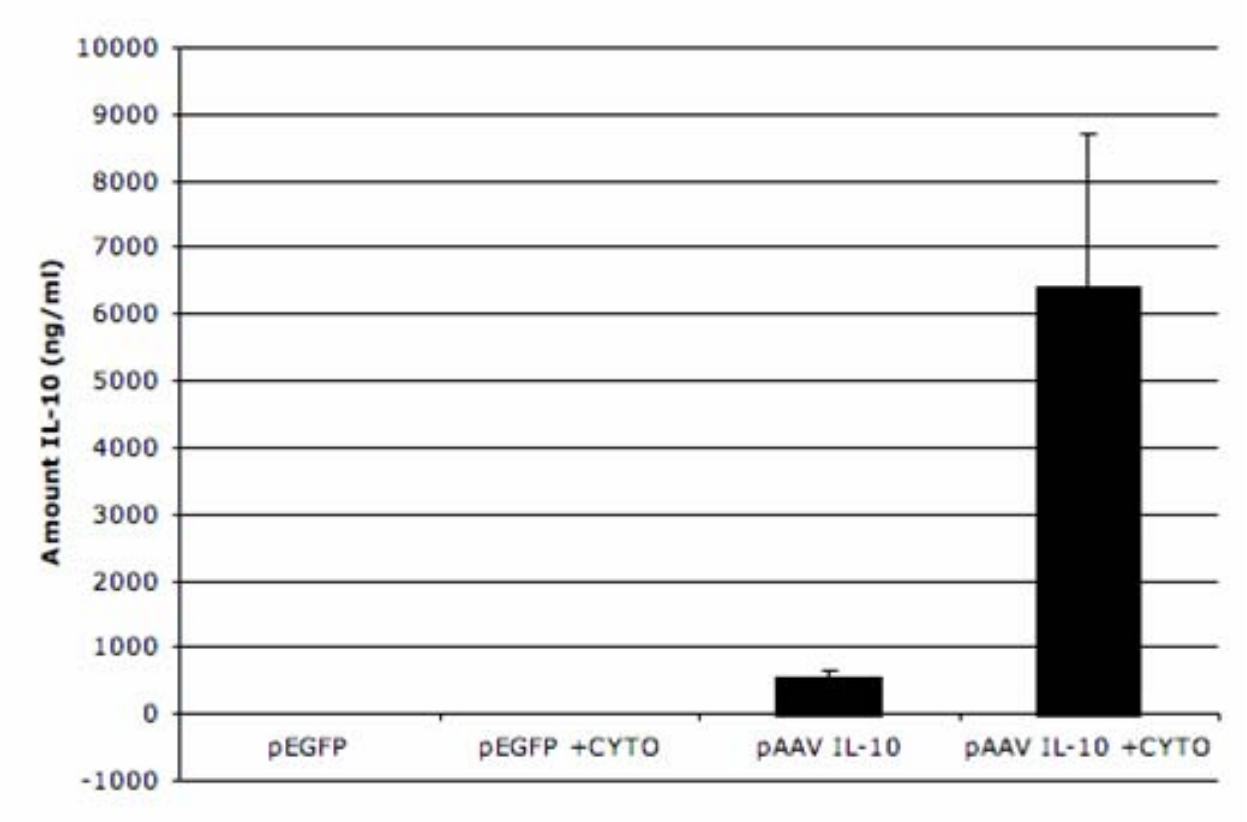


Figure 19: Inflammatory cytokines increase gene expression from stably transfected 293 cells.

293 cells were electroporated with either the plasmid used to construct the dsAAV (IL-10) or a pEGFP (encoding EGFP) control plasmid. Cells were selected for 2 weeks with the anitbiotic G418. $5x10^4$ cells were seeded into 24 well plates in the presence or absence of inflammatory cytokines. After 5 days, the supernatants were collected and tested for IL-10 expression by ELISA. This experiment is representative of two separate experiments.

2.5. Discussion

Although gene transfer is an attractive choice for the treatment of many diseases, including RA, there are many obstacles that must be overcome for it to become a viable treatment option. Obtaining long-term expression and regulation of transgene expression are two of the key demands that have proven especially difficult in regards to gene transfer. Other researchers have successfully regulated transgene expression in the treatment of retinal degeneration and collagen-induced arthritis using tetracycline inducible AAV vectors (57, 284). Previously an inflammation-inducible adenoviral expression system for local treatment of the

joint was developed with similar goals in mind (41). Another approach that has been successful is the use of proteasome inhibitors such as zLLL, which we have shown to increase transgene expression several fold in human, but not mouse, FLS (275). The expression was regulatable in that removal and re-addition of the inhibitor led to repeated increases in transgene expression. Ideally, gene transfer vectors could auto-regulate transgene expression, with expression being turned on during active disease, and then turned off once inflammation is brought under control. This feature is especially desired for treatment of inflammatory diseases like RA that are characterized by repeated disease flares, with intervening periods of relative quiescence. Our studies suggest that AAV possesses these qualities. Transgene expression can be repeatedly re-induced upon addition and removal of inflammatory cytokines.

AAV has been used with some success in the treatment of cystic fibrosis and hemophilia B (285, 286). Unfortunately, AAV, while capable of transducing peri-articular tissues, only minimally transduces both mouse and human synovium (287). For local treatment to be effective using AAV, new methods must be developed to increase transduction of synovium. Thus far, clinical trials have only utilized single stranded versions of AAV. Our data suggest that for inflammatory diseases such as RA, double stranded versions of AAV may prove more effective, especially if they are to be administered locally in the joint.

The ability of proteasome inhibitors to increase transgene expression in FLS also appears to be significantly more effective if double stranded AAV is used (see Chapter 3). Zhang et. al. found a dual therapeutic use of proteasome inhibition in human cystic fibrosis airway epithelia (233). Not only did they observe an increase in CFTR transgene expression, but they also saw a simultaneous inhibition of ENaC currents that was independent of vector administration. In a similar fashion, inflammatory cytokines also appear to have a dual role. They increase AAV

transgene expression in FLS dramatically and are also capable of regulating expression. This makes the local inflammatory environment of the joint in RA ideally suited for providing high levels of transgene expression from AAV vectors.

Our results also suggest that TNFα, IL-1β, and IL-6 may be primarily responsible for the increase in AAV transgene expression observed in the synovium *in vivo* in the presence of inflammation induced by LPS in a rat model of arthritis (207). The presence of inflammation in a human TNFα-transgenic mouse model of arthritis also correlated with increases in the level of transgene expression from AAV vectors (235). Transducibility by AAV is species-specific and mouse fibroblasts are resistant to transduction. In contrast, human fibroblasts are transducible in the presence of either proteasome inhibitor or inflammatory cytokines. Therefore, our findings in human FLS have relevance to the clinical setting. The fact that only synovial fluid from patients with arthritis increases AAV transgene expression also suggests that this phenomenon may occur *in vivo*. Regulation at the level of the CMV promoter, which also drives expression from our AAV vectors, has been implicated in this process. It was also suggested that inflammation-induced activation of NFκB and its subsequent binding to the four consensus binding sites in the CMV promoter and enhancer may be responsible for the observed effects. However, we observed similar findings *in vitro* using AAV vectors under control of the chicken beta actin promoter, suggesting that the effect might not be promoter specific. This did not rule out a role for the CMV enhancer, which is present in both vectors and does contain NFκB elements. We did find that the effect of cytokines could be prevented in cells expressing a super IκB repressor that prevents actiation of NFκB. Therefore, NFκB activation is required for inflammatory cytokines to increase AAV transgene expression. NFκB could act by either

binding directly the promoter/enhancer to turn on transcription of the transgene or by inducing other cellular factors that lead to the increase.

In addition, the proteasome inhibitor zLLL, which is known to inhibit NFκB activation (288), also increases AAV transgene expression in FLS (275), suggesting that other underlying mechanisms, such as effects on mRNA stability, may be at work. Our results showed that transgene mRNA levels increased significantly upon cytokine stimulation, but further studies will need to be performed to determine if the effect is on mRNA synthesis, stability, or degradation. One possibility is that the rise in trangene mRNA could be secondary to altered viral trafficking and/or uncoating and not at the transcriptional or post-transcriptional level. In fact, we observed significant loss of intact capsids in the cytoplasm following cytokine exposure, suggesting an effect on uncoating of the virus.

Interestingly, AAV infection did not appear to be a requirement for inflammatory cytokines to increase transgene expression. Cells stably transfected with plasmid DNA containing the viral genome also demonstrated similar increases in gene expression following exposure to cytokines. This suggests that although cytokines promote uncoating of the virus, their primary effect may be at a step after the viral DNA has entered the nucleus, possibly transcription, translation, or post-translational processing. It should be noted that stably transfected cells have incorporated the plasmid DNA into the host genome, while the viral DNA in AAV-infected cells is thought to remain episomal. What effect this may have on the assay remains unclear. In addtion, the viral ITRs may be involved in this phenomenom and their role requires further exploration.

The combination of all three inflammatory cytokines appears to increase transgene expression from FLS even further at later time points or upon secondary stimulation. Why this is

observed only when all three cytokines are present remains unclear but suggests that long-term exposure of FLS to multiple inflammatory mediators, while contributing to the pathological features of RA, may actually have beneficial effects on AAV transgene expression. This phenomenon could relate to the apparent delay in maximal expression of up to 30 days to 3 months observed *in vivo* with AAV in other studies (54, 139). This again highlights the potential benefits that AAV may have over other gene transfer vectors in the treatment of RA. More studies examining control of expression from AAV vectors will have to be performed to elucidate the mechanisms of this phenomenon.

Expression from AAV vectors has been improved in other cells by modifying the tropism of the virus through manipulation of the AAV capsid protein (149, 221). Our data suggests that most FLS are in fact infected with AAV2, but that some cellular process is inhibiting the ability of the viral transgene to be maximally expressed. One possibility is that nuclear trafficking of AAV is enhanced under inflammatory conditions. In fact, impaired nuclear trafficking of AAV has been shown to limit transduction in murine 3T3 fibroblasts (172). In addition, the PI3K pathway has been demonstrated to be necessary for intracellular movement of AAV to the nucleus (165). TNFα, IL-1β, and IL-6 have all be shown to activate PI3K in various cell types (289-291) and it is possible that PI3K activation could lead to increased trafficking of AAV to the nucleus, with subsequent increases in transgene expression. This hypothesis is supported by our data demonstrating that inhibition of PI3K by wortmannin blocks the increase in AAV transgene expression in response to inflammatory cytokines. Our observation that IL-17 does not increase transgene expression also supports this hypothesis. It has recently been demonstrated that this cytokine does not activate PI3K in human airway epithelium (292). However, the observation that removal of cytokines returns transgene expression to normal

suggests that other mechanisms are likely also involved. The observation that PI3K activation plays a key role in the level of transgene expression from AAV infected FLS suggests a new strategy that may improve AAV expression in other cell types. It should be noted that wortmannin and NFκB inhibition act independently of each other, as treatment of dermal fibroblasts with wortmannin did not inhibit NFκB activation, as measured by an NFκB responsive luciferase reporter gene.

Our results demonstrate that inflammatory cytokines have a remarkable ability to regulate transgene expression in human RA FLS, especially using dsAAV vectors. This could have broad clinical implications, as dsAAV vectors encoding transgenes like sTNFR-Ig could be injected locally in the joint. Inflammatory mediators in the synovial fluid would then increase the expression of the therapeutic transgene. The regulatory nature of AAV transgene expression by inflammation could provide an additional level of control, with transgene expression only turned on during active disease and turned off once disease is brought under control and the level of inflammatory mediators decreases.

3. The proteasome inhibitor zLLL regulates AAV transgene expression in fibroblast-like synoviocytes

3.1. Introduction

Protein degradation is an important part of cellular homeostasis. In eukaryotes, protein degradation occurs in several parts of the cell, including the cytoplasm, nucleus, membrane, endoplasmic reticulum, and lysosomes. Lysosomal degradation by cathepsins and intramembrane proteolysis are important in many celluar processes. However, lysosomes only account for approximately 10-20% of cellular protein degradation. The ubiquitin-proteasome system (UPS) accounts for 80-90% of protein degradation and is the focus of this discussion (265). In this system, proteins are covalently tagged with ubiquitin by the ubiquitin-conjugating

enzymes E1, E2, and E3. The poly-ubiquitin tag targets the protein for degradation by the 26S proteasome, a giant protease. Through ATP-dependent and independent steps, the protein is sequentially unfolded and cleaved into peptides and amino acids that can be re-utilized by the cells (293).

The 26S proteasome is made up of a catalytic 20S structure containing the proteolytic core and a 19S cap structure that contains regulatory functions. The 20S core is made up of 4 heptameric stacked rings of α and β type subunits. Eukaryotic proteasomes have 3 major peptidase activities, chymotrypsin-like, trypsin-like, and caspase-like (293).

The UPS is important in many cellular processes and, therefore, the proteasome has become an attractive drug target. Several classes of proteasome inhibitors have been discovered and are summarized in Table 2. One peptide boronate in particular, bortezomib, is the first proteasome inhibitor evaluated in clinical trials and was recently approved for use in the treatment of multiple myeloma (294). It also shows promise in the treatment of other hematologic and solid malignancies. The primary clinical toxicities were thrombocytopenia and electrolyte abnormalities, although both were manageable (295, 296).

Peptide aldehydes such as N-acetyl-Leu-Leu-Norleucinal (calpain inhibitor I) and N-acetyl-Leu-Leu-Leucinal (zLLL or MG132) reversibly inhibit the 20S proteasome and act by modifying the catalytic hydroxyl group of threonine by forming a hemi-acetal bond. They inhibit the chymotrypsin-like activity of the proteasome and decrease cytosolic protein degradation (297). Peptide boronates form tetrahedral adducts with an N-terminal threonine of the 20S proteasome. Peptide vinyl sulfones covalently modify a catalytic threonine present in the β proteasome subunits (265). Peptide epoxyketones act on a catalytic N-terminal threonine residue of the proteasome to form an irreversible morpholino derivative (298). Lactacystin

inhibits chymotryptic activity of the proteasome by covalently binding to an N-terminal threonine of the β5 proteasome subunit via an ester bond (299).

Table 2. Types of proteasome inhibitors and their mechanism of action.

Type	Members of Class	Mechanism
Peptide Aldehydes	MG132 (zLLL) Calpain Inhibitor I	Modify catalytic hydroxyl group of 20S proteasome by forming a hemiacetal bond
Peptide Boronates	Bortezomib MG262	Form tetrahedral adduct with active site N-terminal threonine of 20S proteasome
Peptide Vinyl Sulfones	NLVS YLVS	Covalently modify catalytic threonine of β subunits
Lactacystin and Derivatives	Lactacystin Clasto-lactacystin-β-lactone	Covalently binds N-terminal threonine of β5 subunit and forms an ester bond
Peptide Epoxyketones	Dihydroeponemycin Epoxomicin	Form an irreversible morpholino derivative with N-terminal threonine of proteasome

The use of proteasome inhibitors results in accumulation of protein aggregates into "aggresomes", which consist of ubiquitinated proteins and components of the 20S proteasome. Proteasome inhibition can also inhibit NFκB activity and, if maintained long enough, will eventually lead to cellular apoptosis (293).

In HeLa cells, both AAV2 and AAV5 capsids appear to be substrates for ubiquitination and subsequent degradation by the proteasome. However, it appears that the capsid must first be modified in the endosome before it can be conjugated with ubiquitin, as intact virions are not substrates for ubiquitination in vitro. The exact nature of this modification remains unknown

(175). This finding led to several other studies examining the role of the UPS system in AAV transduction of several cell types.

Two separate classes of proteasome inhibitors, tripeptidyl aldehydes and anthracycline derivatives, can synergistically augment AAV transduction in human airway epithelia, suggesting these different classes of inhibitors modulate different proteasome functions that mediate AAV transduction (230). In polarized human airway epithelia, the proteasome inhibitor LLnL was able to increase transgene expression after apical infection with AAV2 or AAV5 vectors. Interestingly, basal infection was unaffected by the presence of the proteasome inhibitor. Both wild type or self-complimentary vectors provided similar expression levels, suggesting that second-strand synthesis is not a rate-limiting step of AAV transduction following apical infection of polarized human airway epithelia (231). *In vivo*, proteasome inhibitors increased transduction in the mouse lung from undetectable to 10% expression in epithelial cells of the large bronchioles of the lungs. Liver expression also increased 10-fold upon proteasome inhibition. Muscle transduction, however, was unaffected, suggesting cell type specific effects of proteasome inhibition (174).

Proteasome inhibitors also increase transgene expression in human intestinal epithelial cells *in vitro* up to 400-fold (232). A dual therapeutic use of proteasome inhibitors in human cystic fibrosis airway epithelia infected with AAV has been observed. Not only was there an increase in CFTR transgene expression, but there was also a simultaneous inhibition of ENaC currents that was independent of vector administration (233). The following studies examine the role of proteasome inhibition on AAV-mediated transgene expression in fibroblast-like synoviocytes.

3.2. Materials and methods

3.2.1. Isolation of fibroblast-like synoviocytes (FLS)

FLS were isolated as described in the materials and methods section of chapter 2.

3.2.2. AAV production

The vectors AAV (mIL-4), encoding the murine IL-4 cDNA, AAV(mIL-10), encoding the murine IL-10 cDNA, and AAV(EGFP), encoding the green fluorescent protein (GFP) cDNA, were driven by the CMV promoter. All AAV constructs were derived from AAV2 and were generated by either 293/triple transfection or B50/hybrid method (300). Adenovirus helper functions were supplemented by pAdORF6, a plasmid construct carrying all essential adenovirus helper genes (300). Transfection was carried out using the standard calcium phosphate precipitation method. AAV vector preps were purified by CsCl gradient centrifugation (301). The genome titers of vector preps were determined by the real time quantitative PCR method (300), whereas the transducing titers of vector preps were assayed on 84-31 cells as described elsewhere (302). Virus was stored at –80^{o}C in buffer containing 20 mM Tris pH 7.4, 1 mM $MgCl_2$, 150 mM NaCl, and 10% (v/v) glycerol.

3.2.3. Recombinant adenovirus

Ad(BglII) is an E1a-E3-deleted replication-defective adenovirus type 5 backbone vector lacking a transgene and was generously provided by J.A. Bluestone and J.M. Leiden. Recombinant adenovirus was produced and propagated in 293 cells and purified by cesium chloride density centrifugation, as previously described (303, 304). Virus plaques were purified three times before the production of seed stocks, and their identities were confirmed by restriction endonuclease digestion and DNA sequence analysis. Viral titers (particles per ml) were calculated by $OD260X10^{12}$ following lysis of viral stocks in 0.1% SDS, 10 mM Tris-HCl

(pH 7.4), and 1 mM EDTA at 56˚C for 10 min. Virus was stored at –80°C in buffer containing 10 mM Tris pH 7.4, 1 mM $MgCl_2$, 10% (v/v) glycerol.

3.2.4. *In vitro* infection

Infection of cells was carried out as described in the materials and methods section of chapter 2. In some cases, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (zLLL, also called MG-132, purchased from Calbiochem, La Jolla, CA) was added for 24 hours either at the time of infection or at later time points. zLLL was prepared as a 20 mM stock solution in DMSO and diluted in RPMI containing 10% fetal calf serum prior to use at a working concentration of 20 or 40μM as indicated.

3.2.5. Assays for mIL-4 and mIL-10

Concentrations of mIL-4 and mIL-10 in cell supernatants were determined by enzyme-linked immunosorbent assay (ELISA), as previously described (56). Plates were washed and developed with ABTS peroxidase substrate (Kirkegaard & Perry) and read at 410 nm with a Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA). Assays were performed in duplicate wells and the results averaged. In all cases, duplicates differed by <5%.

3.2.6. Flow cytometry

Flow cytometry was performed as described in the materials and methods section of chapter 2.

3.2.7. Analysis of nuclear and cytoplasmic AAV DNA

Human fibroblast-like synoviocytes infected with $1x10^4$ particles per cell of rAAV(IL-10) were collected at various time points, washed with PBS, and resuspended in 0.5ml lysing buffer (1.3M sucrose, 20 mM $MgCl_2$, 4 mM Tris, 4.2% Triton X-100) and 1.5ml ice cold water and incubated on ice for 10min. The nuclei were pelleted by spinning at 1400rpm for 15min at

4°. The supernatant containing the cytoplasmic fraction was transferred to a new tube and the nuclear pellet was washed with 2 ml PBS. Both tubes were spun again at 1400rpm for 15min. The cytoplasmic fraction was transferred to a new tube and 25µl of RNaseA (200 U/ml) was added. Viral nuclear DNA was isolated using a Qiagen plasmid maxi kit. After isolation, both the cytoplasmic and nuclear fractions were treated with 50µl Proteinase K (Invitrogen, 10mg/ml) and incubated at 50°C for 1 hour. The samples were cooled to room temperature and 10µl of glycogen (Boehringer Mannheim, 20mg/ml) was added. The DNA was precipitated with isopropanol, washed with 70% ethanol, air-dried, and resuspended in water. Successful isolation of the cytoplasmic and nuclear fractions was confirmed using a cytoplasmic phosphatase assay and western blotting for histone H3, as previously described (172), and by PCR. Cytoplasmic and nuclear DNA fractions were applied to nitrocellulose using a Bio-Dot SF Microfiltration Apparatus (Biorad). A 500 bp biotinylated probe specific for a region within the mouse IL-10 transgene was prepared using the Random Prime DNA Labeling System with biotin-14-dCTP (Invitrogen). Hybridization, washing, and blocking were performed according to the manufacturers instructions. A 1:2000 dilution of SA-HRP (Zymed) was applied. Supersignal West Femto Maximum Sensitivity Substrate (Pierce) was added and the blot was exposed to X-OMAT film (Kodak).

3.2.8. Immunohistochemistry

Immunohistochemistry for intact AAV particles was performed as described in the materials and methods section of chapter 2.

3.3. Results

3.3.1. The proteasome inhibitor zLLL increases AAV transgene expression in fibroblast-like synoviocytes

For local treatment of arthritis to be successful using AAV, the cells lining the joint must be efficiently transduced by the vector. The synovial lining normally consists of a 2-3 cell layer thick tissue made up of macrophage-like and fibroblast-like synoviocytes. During arthritis, these cells proliferate to form a pannus of tissue that secrete molecules that contribute to cartilage invasion and bone destruction. In particular, the role of fibroblast-like synoviocytes in this process has been thoroughly studied. The role of macrophage-like synoviocytes in this process is less well defined, primarily because of the difficulty of isolating and maintaining these cells in culture. Fibroblast-like synoviocytes were isolated from human RA tissue and the ability of both ssAAV and dsAAV to transduce these cells was examined. Both transduced the cells only minimally, although dsAAV provide for significantly more transduction than ssAAV (Figure 20A and B). The addition of the proteasome inhibitor zLLL increased transgene expression dramatically with both vectors, from 2.3% to 23% for ssAAV and from 15.7% to 72.2% for dsAAV (Figure 20C and D), although transgene expression returned to baseline several days after removal of the inhibitor (data not shown).

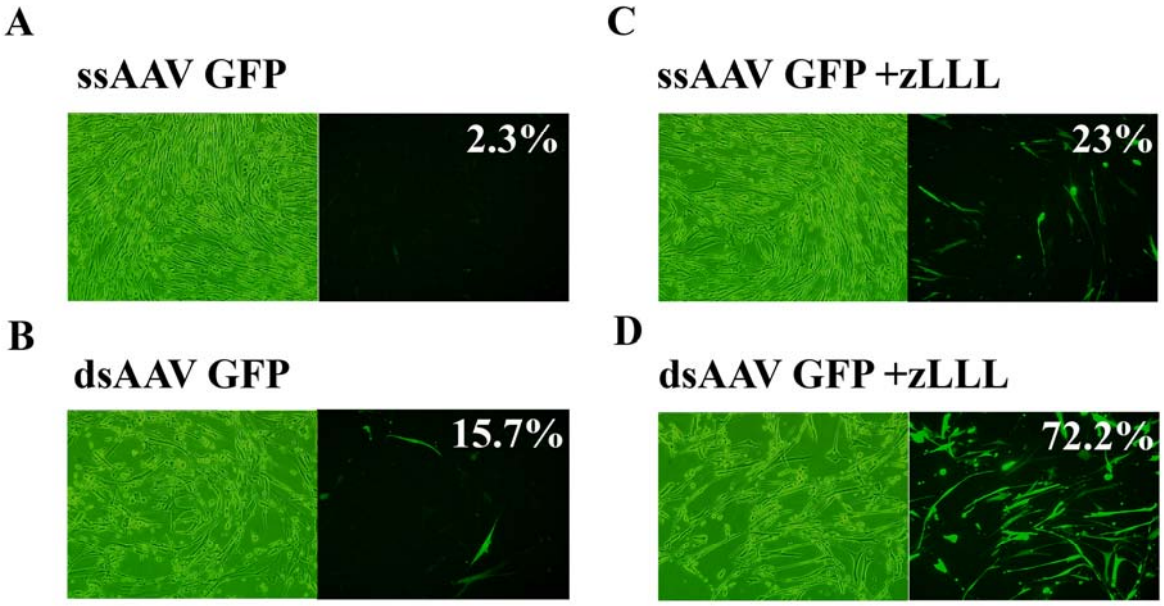


Figure 20: The proteasome inhibitor zLLL increases AAV-mediated transgene expression in fibroblast-like synoviocytes.

Human RA FLS were infected with either ssAAV or dsAAV encoding GFP at $1x10^4$ particles/cell. The proteasome inhibitor zLLL (20μM) was added, as indicated, for 24 hours and then washed out. Cells were then analyzed by flow cytometry 24 hours later. The left part of each panel shows a field of cells as visualized by light microscopy. The right part of each panel represents the same field, this time as visualized by fluorescent light. GFP positive cells are green. The percent positive cells, as measured by flow cytometry, are indicated. This experiment is representative of three separate experiments.

3.3.2. Proteasome inhibitor can regulate transgene expression in human RA FLS *in vitro*

We were surprised to observe that the zLLL-induced increase in transgene expression was transient, such that after removal, expression returned to baseline. This is inconsistent with the proposed mechanism of action of zLLL, that of increasing viral trafficking to the nucleus, as transgene expression should be stable once the viral genome reaches the nucleus and sufficient second strand synthesis occurs. To further explore this finding, synovial cells were re-exposed to zLLL following the loss of transgene expression, this time using a ssAAV vector encoding mouse IL-10. Transgene expression could be repeatedly induced by re-exposure to zLLL

(Figure 21A). In each case, expression peaked 2-3 days following exposure to zLLL and then dropped off. We were able to re-induce transgene expression as far out as 32 days after infection. This is in contrast to co-infection with Ad(BglII), a recombinant adenovirus lacking a transgene, which resulted in a stable increase in transgene product (Figure 21B).

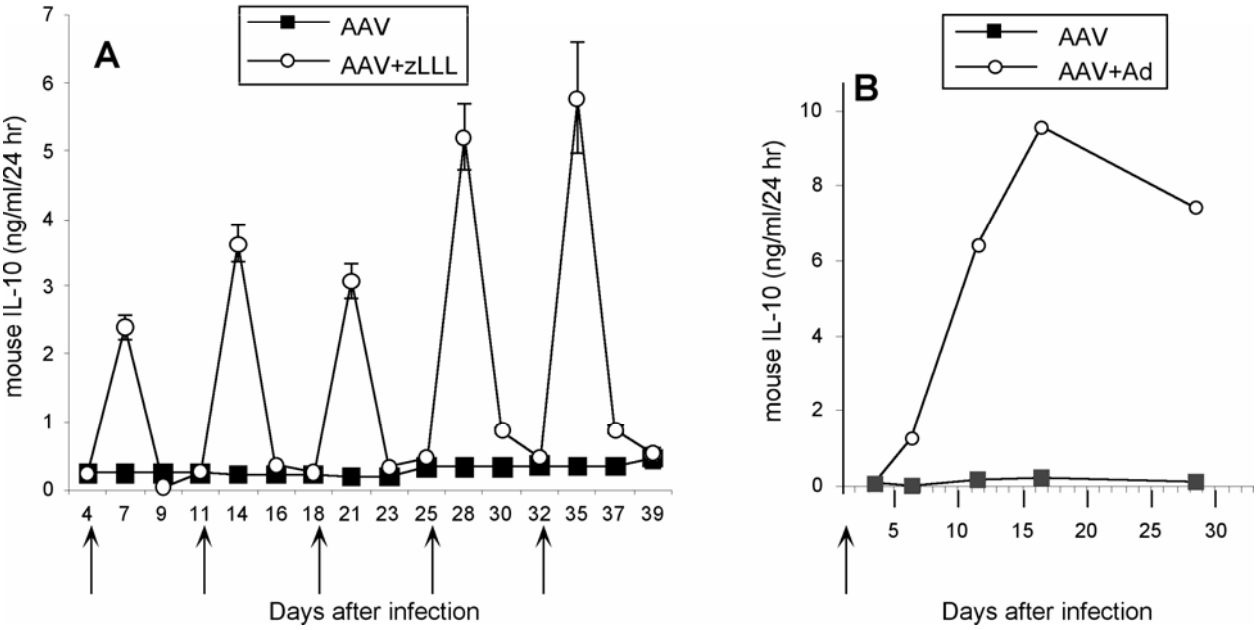


Figure 21: zLLL can regulate rAAV-mediated transduction of RA FLS in vitro.

RA FLS were infected with $1x10^4$ particles/cell of ssAAV(mIL-10) in the presence or absence of A) 20 μM zLLL or B) 100 particles/cell of the recombinant adenovirus, Ad(BglII), which lacks a transgene. Arrows indicate exposure to zLLL or adenovirus (Ad). The supernatants were assayed by ELISA for mIL-10 on the days indicated. Each time point represents the amount of transgene product secreted into the supernatant over a 24 hour period.

3.3.3. Proteasome inhibition leads to increased trafficking of AAV viral DNA to the nucleus

Since proteasome inhibitors are known to increase nuclear trafficking of AAV DNA in some cell types, the effect of zLLL on AAV transport was investigated. Cytoplasmic and

nuclear fractions were isolated from AAV-infected cells at various time points prior and after exposure to zLLL and the fractions were analyzed for the presence of AAV DNA (Figure 22). The majority of the AAV DNA was located in the nucleus within 4 hours of infection. However, at 3 days post-infection, increased DNA was noted in the cytoplasm, apparently due to trafficking of AAV DNA between the nucleus and the cytoplasm. Addition of zLLL led to a transient loss of AAV DNA from the cytoplasm that was maximal at 24 hours and was followed by a return of AAV DNA to the cytoplasm. A second exposure to zLLL 7 days later again resulted in a similar transient loss of cytoplasmic AAV DNA. This observation supports the role of proteasome inhibitor in enhancing transport of AAV DNA to the nucleus. However, there was no obvious correlation between nuclear DNA and either RNA or protein levels of the transgene product. For instance, transgene DNA levels in the nucleus were similar before and after a second exposure to zLLL (Figure 22) even though amount of transgene product was markedly different (Figure 21).

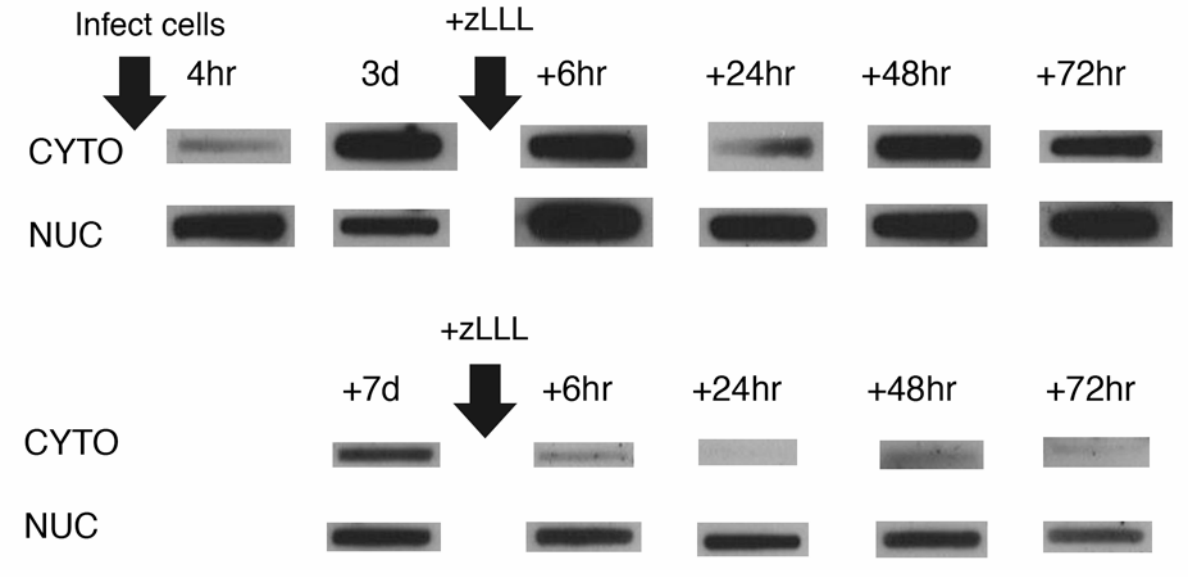


Figure 22: Effect of zLLL on AAV DNA trafficking.

RA FLS were infected with $1x10^4$ particles per cell of AAV(IL-10). zLLL was added 3 days after infection and again 7 days later. Cytoplasmic and nuclear DNA was isolated at the indicated time points. The first two time points are relative to the time of infection and all subsequent time points are relative to the addition of zLLL. Slot blot analysis using a transgene-specific biotinylated probe was used to detect the viral DNA in each fraction. The probe did not bind to DNA from non-infected cells (not shown). The studies were repeated to ensure reproducibility, with similar findings.

3.3.4. Proteasome inhibition leads to aggregation of intact AAV particles in the perinuclear area

Next we wanted to determine if proteasome inhibition had any effect on the intracellular localization of intact AAV viral particles. AAV-infected cells were treated with the proteasome inhibitor zLLL. 24 hours after zLLL removal the cells were fixed and stained for intact AAV particles. Treatment with the proteasome inhibitor led to a distinct and intense primarily peri-nuclear staining (Figure 23). The intense staining is not surprising because normally the AAV capsid is ubiquitinated and subsequently degraded by the proteasome (175). Inhibition of the proteasome would naturally lead to a buildup of ubiquitinated proteins. Proteasome inhibition has also been reported to lead to the formation of aggresomes and sequestosomes, which are

formed as depots for storage of ubiquitinated proteins when proteasome function is impaired (305). It seems likely that the viral particles are being stored within these aggregates. Intracellular localization of intact AAV particles was examined over several rounds of proteasome inhibition. The total number of aggregates clearly decreased over time, but intact paricles were detected at all time points examined (data not shown). Rarely could any intact particles be observed in the nucleus, consistent with previous studies suggesting that uncoating occurs prior to or during nuclear translocation of the virus (176).

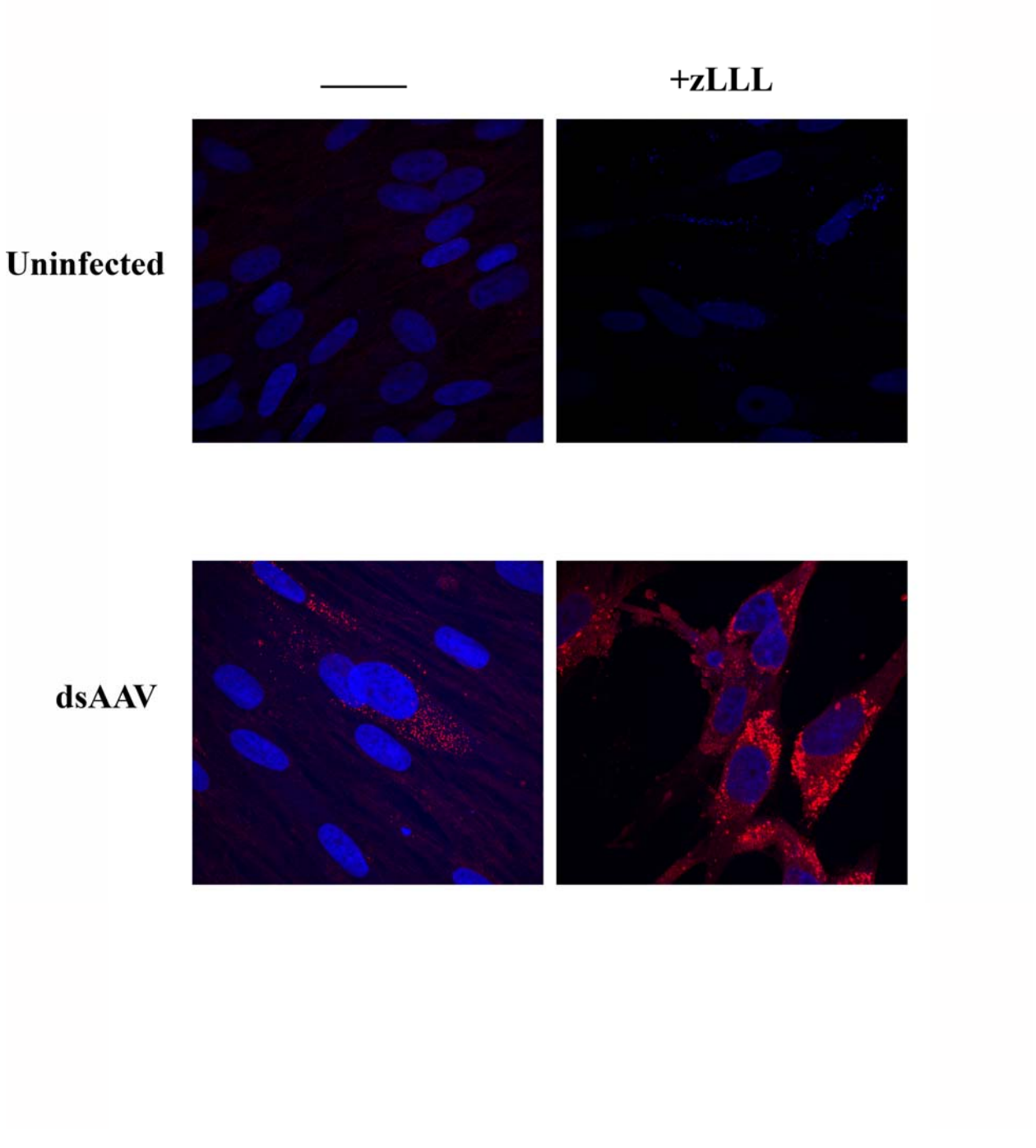


Figure 23: Effect of zLLL on cellular localization of intact AAV particles.

Dermal fibroblasts were seeded onto coverslips. The next day, cells were infected with $1x10^4$ particles/cell of dsAAV (sTNFR-Fc) as described. 24 hours later zLLL (20μM) was added, as indicated. The next day cells were fixed and stained for intact AAV particles as described in the materials and methods.

3.3.5. NFκB inhibition does not prevent the effects of zLLL on AAV transgene expression in dermal fibroblasts

Although zLLL has been demonstrated to inhibit NFκB, we wanted to confirm that inhibition of this transcription factor did not affect the ability of zLLL to increase AAV-mediated transgene expression. A retroviral vector was used to deliver a super IκB inhibitor to dermal fibroblasts and inhibition of NFκB was demonstrated using an NFκB reporter plasmid (see chapter 2). The cells were then infected with dsAAV and the ability of zLLL to increase transgene expression was examined. NFκB inhibition had no effect on the ability of zLLL to increase transgene expression (Figure 24). This suggests that proteasome inhibition with zLLL increases AAV-mediated transgene expression in an NFκB-independent manner.

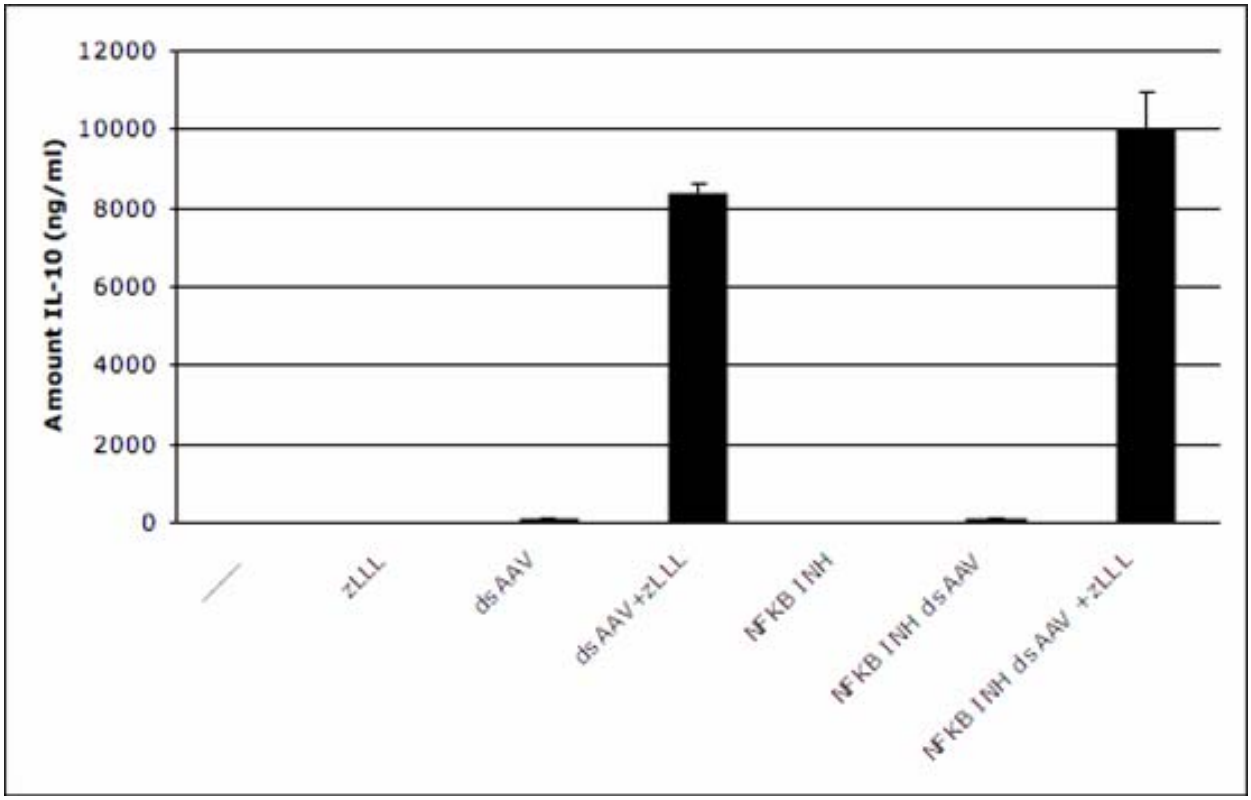


Figure 24: Effect of NFκB inhibition on ability of zLLL to increase AAV trangene expression in dermal fibroblasts.

Hs69 dermal fibroblasts were seeded in a 24 well plate at $5x10^4$ cells per well. The following day the cells were infected with dsAAV (IL-10). 24 hours later the proteasome inhibitor zLLL (20μM) was added. zLLL was removed the next day and supernatants were collected 48 hours later. Transgene expression was measured by ELISA. This is a preliminary result and the experiment has been performed only two times.

3.4. Discussion

The proteasome represents the major non-lysosomal process for degradation of proteins by ATP/ubiquitin-dependent proteolysis. The majority of proteins in mammalian cells are hydrolyzed by a distinct pathway that requires ATP and the proteasome particle. In this pathway, which is present in both the nucleus and the cytosol, most substrates are first marked for degradation by covalent linkage to multiple molecules of ubiquitin (306). This includes certain transcription factors and rate-limiting enzymes.

In our studies using human FLS, we saw accumulation of the majority of AAV viral DNA in the nucleus within 4 hours after infection. Interestingly, three days after infection there appeared to be a shift of some of the viral DNA back to the cytoplasm. This is similar to the effect that Xiao et. al. saw in AAV-infected HeLa cells in the presence of adenovirus co-infection (169). Although they dismissed this observation as being due to disruption of nuclei caused by adenovirus cytopathology, our data suggest that this phenomenon may be real. Even more suggestive is the fact that multiple additions of zLLL appear to shift AAV viral DNA from the cytoplasm back to the nucleus soon after addition, with some viral DNA once again returning to the cytoplasm after 48hrs. However, it is also possible that zLLL could also disrupt the nuclei as well. This may be especially relevant because the viral particles appear to be localized in a primarily peri-nuclear location. For example, during isolation of the cytoplasmic and nuclear fractions of the cell zLLL may make the nucleus "sticky," leading to the appearance of viral DNA in the nuclear fraction.

This shifting effect might account for some, but not all of the increase in transgene seen upon zLLL addition, since 72 hrs after proteasome inhibition, when maximum transgene protein is observed, the levels of viral DNA in the nucleus are comparable to the levels present prior to zLLL addition. Our data shows that zLLL also effects transgene expression at the level of gene transcription and/or mRNA stability. Since nuclear translocation of AAV has previously been shown to be independent of the nuclear pore complex, one possibility is that AAV virons and/or DNA are stored in a perinuclear compartment that may fuse with the nuclear envelope upon zLLL addition, thereby releasing its contents into the nucleus. In the same fashion, a mechanism for transporting the viral DNA back out of the nucleus could also exist. The sequestration of viral particles into aggresomes could somehow contribute to this phenomenon, but further

studies on the nuclear trafficking of AAV in human FLS and other cell types are needed to fully understand these observations.

The present study demonstrates that the AAV-mediated transgene expression in human RA FLS can be dramatically enhanced by proteasome inhibition. The most striking observation was that transgene expression could be regulated by repeated exposure to proteasome inhibitors. This was surprising, in light of previous studies suggesting that proteasome inhibitors enhance AAV-mediated transgene expression by increasing nuclear accumulation of AAV particles and DNA (174, 307). While nuclear transport of AAV is likely to partly explain the effect of proteasome inhibition, the data presented here suggest that there must be additional mechanisms at work. If proteasome inhibitors acted solely by increasing nuclear accumulation of AAV DNA, then once the cells were exposed to the proteasome inhibitor expression is likely to be relatively stable. However, proteasome inhibitors induced a transient and repeatable increase in transgene expression. The effect was observed even when the proteasome inhibitor was added 2 weeks after infection, suggesting a point of action downstream of binding and internalization of the AAV particle.

Douar et. al. demonstrated a 25-50-fold increase in transduction efficiency of HepG2 and HeLa cells in the presence of zLLL (307) and concluded that a significant portion of AAV particles are degraded by the proteasome. It is unclear, however, how the proteasome could degrade intact viral particles, as they are too large to fit into the proteasome structure and would need to first be degraded into proteins that could be ubiquitinated. Proteasome inhibitors may however increase the concentration of a protein that enhances nuclear transport or AAV. Thus, one possibility is that the AAV is harbored in a stable cytoplasmic compartment and that a portion is released and transported to the nucleus upon each exposure to the proteasome

inhibitor. However, this would not explain the transient nature of expression for the AAV that reached the nucleus, unless the AAV genome is unstable in the nucleus. A more likely explanation is that the AAV genome is transported to the nucleus in an inactive form until acted upon, either directly or indirectly, by the proteasome inhibitor. Proteasomes have been found to degrade numerous transcription factors, which is a likely mechanism to explain the results described here. Potential effects of proteasome inhibitors on protein regulators of gene transcription are of particular interest. For example, such molecules have been shown to influence NFκB-dependent gene transcription by blocking the degradation of IκB (308). Alterations in tumor suppressor genes, such as p53 (309) and PTEN (310) have also been described. The correlation of enhanced protein expression with increased transgene mRNA suggests an effect on transcription, although enhanced RNA stability is also a possibility. The effect was observed with different transgenes and different promoters, suggesting that this is not idiosyncratic but a general phenomenon. However, our results demonstrated that the effects of zLLL are NFκB-independent. This indicates that if the effects of zLLL are at the level of a transcription factor, some other factor must be involved. Studies are currently underway to further elucidate the mechanism of action of proteasome inhibitors on AAV-mediated transgene expression in RA FLS.

As one of the key safety issues in the application of gene therapy is the ability to control and regulate transgene expression, the finding that proteasome inhibitors can regulate expression in AAV-transduced RA FLS *in vivo* suggests a possible approach to the use of AAV in arthritis. One potential complication to such an approach is that an arthritic joint is characterized by infiltration with inflammatory cells, including neutrophils, lymphocytes, and monocytes. While such cells are generally poorly permissive for AAV, it is possible that administration of AAV

into an inflamed joint might lead to infection of these inflammatory cells, in addition to synoviocytes. It is unclear whether transgene expression in such cells would be influenced in the same way by proteasome inhibitors. The degree to which inflammatory cells in the joint are transduced by AAV, and the effect of proteasome inhibition on transgene expression in these cells, would need to be determined before such an approach should be tested in the clinical setting. Proteasome inhibitors have been administered to patients as an experimental therapy for cancer without substantial toxicity (311). Their use in a chronic disease such as RA would require further investigation.

4. Inter- and intra-species variation in transduction of fibroblast-like synoviocytes using different AAV serotypes

4.1. Introduction

AAV is a small parvovirus with broad tissue tropism. In human and non-human primate tissues eleven different serotypes of AAV (1-11) have been isolated and characterized. AAV has also been isolated from several other species, including cow, chicken, sheep, snake, lizard, and goat (121).

The first step in AAV infection is viral binding to the cell surface via high affinity cellular receptors. Different serotypes of AAV infect different cell types based on the availability of their respective receptors and co-receptors. Following infection, the virus must efficiently traffic to the nucleus before second-strand sythesis and transcription can occur. Defects in any point along this pathway can prevent efficient transduction of the cell. This is demonstrated by the wide variability in the tropism of the different serotypes of AAV in different cell types and in different species. These differences occur both between and within species.

Individual species demonstrate striking differences in transduction with different serotypes of AAV. AAV6 gave more efficient transduction of mouse epithelial cells in both

large and small airways than AAV2 (144). When injected into day 15 fetuses *in utero*, AAV5 encoding luciferase provided higher expression than AAV2 when the mice were examined 15 months after birth (145). Following sub-retinal injection in mice, AAV5 proved more efficient in transducing photoreceptor cells than AAV2 (146). AAV2, AAV4, and AAV5 demonstrated different tropism after injection into the mouse central nervous system. AAV4 and 5 gave 10-fold more transgene positive ependymal cells than AAV2 afer injection into the lateral ventricle of mice. After striatal injection, AAV4 demonstrated mainly ependymal cell transgene expression, but no expression was detected in the parenchyma. In contrast AAV2 and 5 both had transgene expression in parenchymal cells following striatal injection, with AAV2 transgene expression confined mainly to neurons, while for AAV5 transgene expression was detected in both neurons and astrocytes. In addition, 15 weeks after injection, AAV5 gave 5000-fold higher expression than AAV2 (147).

There were distinct patterns of transduction in the gerbil hippocampus using either AAV2 or AAV5, with AAV2 infecting pyramidal and granular cells and AAV5 infecting primarily granular cells (148). Differential transduction and cell tropism was also observed after injection of AAV serotypes 1, 2, and 5 into different regions of the rat brain (149). AAV3 could infect human myeloid and megakaryocytic cells that were resistant to infection with AAV2 (150).

A thorough examination of AAV2 transduction in primary human cells demonstrated significant cell-specific differences in transduction and duration of transgene expression. In particular, skeletal muscle and hair-follicle cells appeared suited for long-term expression of AAV. In some cell types, expression improved over time. In other cell types, expression went down over time. There was an inverse relationship between long-term trangene expression and

the proportion of cells in the G2/M phase, consistent with the idea that most of the viral genome is present as episomes that are diluted out between cells as they divide (151).

There was also significant donor variation in AAV2 transduction in bone marrow derived CD34-positive cells of healthy volunteers. There was significant variability in transgene expression depending on the level of cellular differentiation. This has implications for the use of AAV in gene therapy involving hematopoietic stem/progenitor cells (152).

Just as there are variations in the tropism of different serotypes of AAV within an individual species, an individual serotype of AAV can display different tissue specificity between species. This is important because the results of gene transfer studies in animal models using AAV may suggest one serotype is more suitable for study than another. However, the corresponding human tissue may or may not display similar transduction with the serotype under consideration. This is especially important if the vector is being developed for use in clinical trials for the treatment of human diseases.

Mouse and human airway epithelia demonstrate species-specific differences in AAV transduction, highlighting that the serotype of AAV best suited for animal model studies may not be the same serotype that will work best in human and nonhuman primate models. AAV2 and AAV5 gave approximately equal transduction of human airway epithelia, while AAV5 gave much higher transduction than AAV2 in mouse lung (153). Cross-packaging of AAV2 vector genomes with viral capsids from different serotypes demonstrated that the resulting viruses enabled transduction with broad and differing specificity. In addition, it was readily apparent that different target tissues of rats and mice had a different hierarchy of serotypes for efficient transduction (154).

Bovine AAV behaves in a similar fashion. In a murine salivary gland model bovine AAV gave 11 times more efficient transduction than AAV2 (312). This suggests that development of new vectors for gene transfer using AAV from different species may deomonstrate success, especially in models where the target cells are less permissive for AAV infection. The following studies were designed to examine the transduction of fibroblast-like synoviocytes isolated from different species with different serotypes of AAV, including the novel serotype AAV2.5.

4.2. Materials and Methods

4.2.1. Cell isolation and culture

Human RA FLS, mouse FLS, rabbit FLS, and horse FLS were all isolated as previously described (275, 287, 313, 314).

4.2.2. AAV infection

Cells were seeded at a density of $5x10^4$ cells per well in a 24 well tissue culture plate in RPMI media. The next day the cells were infected with $1x10^4$ particles per cell of dsAAV encoding EGFP with expression driven by the CMV promoter in a total volume of 0.4 ml. After a 6 hour infection, the cells were washed twice with PBS and fresh RPMI media was added. dsAAV was produced as described in the materials and methods section of chapter 2. The following day the proteasome inhibitor zLLL (Calbiochem) was added to the indicated cells at a 20 μM concentration. zLLL was removed 24 hours later, the cells were washed twice with PBS, and 1 ml of fresh media was added. The next day the cells were trypsinized and analyzed by flow cytometry for EGFP expression as described in the materials and methods section of chapter 2.

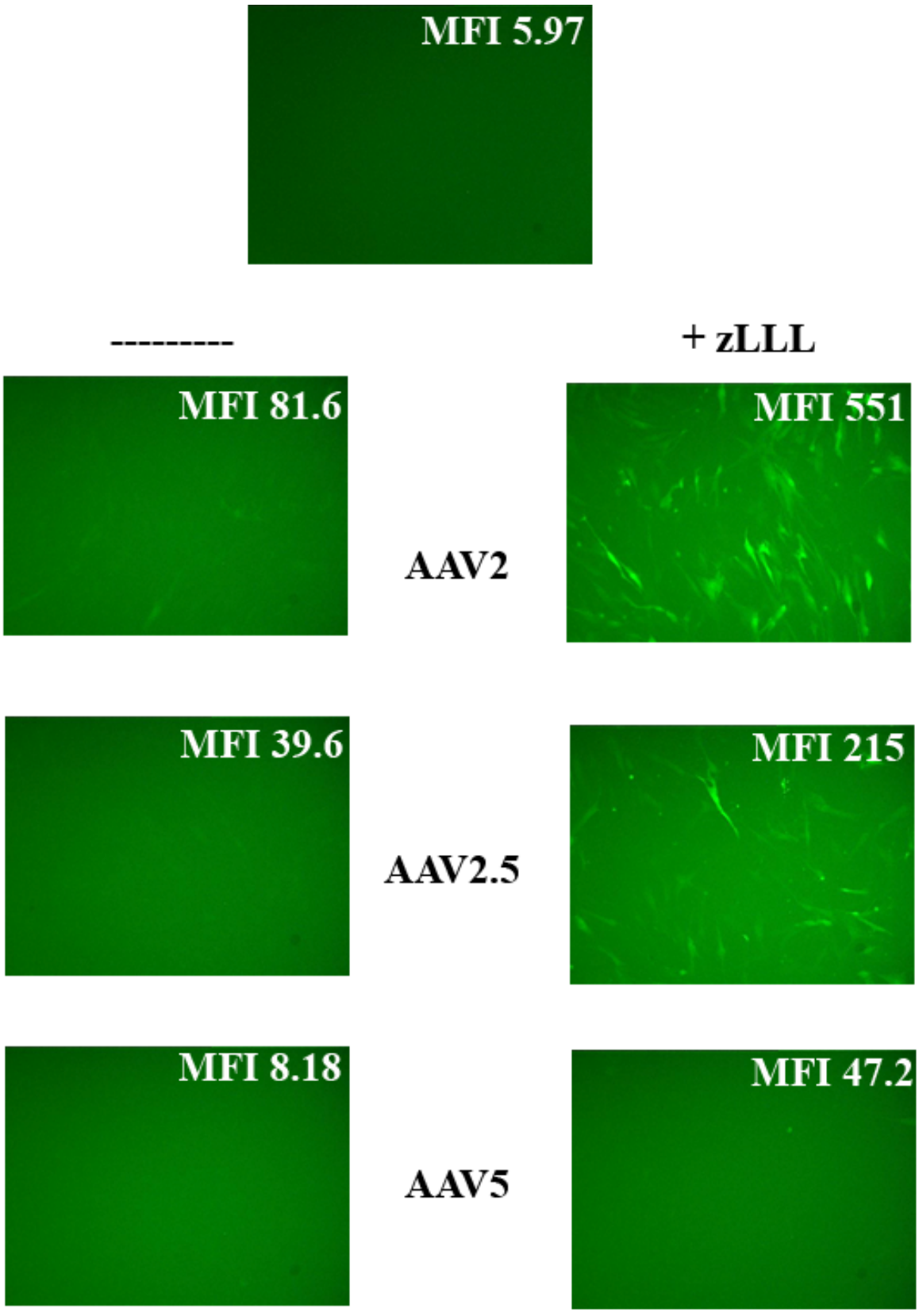
4.3. Results

The ability of 3 different serotypes of AAV (2, 2.5, and 5) to transduce fibroblast-like synoviocytes from human, mouse, horse, and rabbit was examined. AAV 2.5 is a chimeric virus in which the capsid of the virus differs by 5 amino acids from the capsid of AAV2. The virus was originally designed to improve transduction of muscle. The 5 differing amino acids in the chimeric virus contains the muscle-tropic residues of AAV1. The chimeric virus has demonstrated increased transduction of murine muscle and is currently in a phase I clinical trial for the treatment of Duchenne muscular dystrophy. Interestingly, the immune response profile to this chimeric virus resembles that of AAV1, despite the limited changes to the AAV2 capsid (315).

Human RA FLS were only minimally transduced by all three serotypes of AAV tested (Figure 25). In order to amplify the gene expression for a better comparison of the transduction of FLS, the proteasome inhibitor zLLL was added. The effects of zLLL on AAV transgene expression are discussed in chapter 3. As expected, zLLL provided a much clearer picture of which serotype of AAV transduced the synoviocytes most efficiently. For human RA FLS, AAV2 provided the highest level of transgene expression (Figure 25). AAV2.5 provided the highest level of transgene expression in mouse and rabbit FLS (Figure 26 and Figure 27). Horse FLS, like human RA FLS, were transduced most efficiently by AAV2 (Figure 28). The proteasome inhibitor zLLL routinely increased transgene expression 3-7 fold in the FLS cell lines from all four species. It is also important to note that the effects of zLLL were similar with all three of the AAV serotypes tested. The level of expression from all four FLS cell lines using all 3 serotypes in the presence or absence of zLLL is summarized in Figure 29.

Interestingly, horse FLS provided the highest transgene expression by far with all 3 serotypes of AAV tested. Although most gene transfer studies performed to date in mouse and

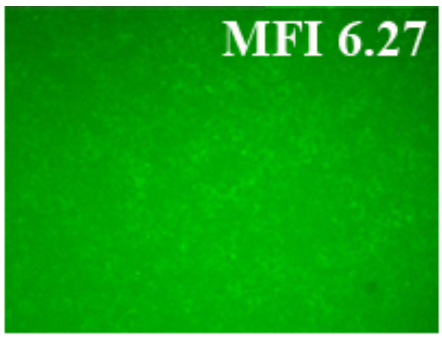
rabbit models of arthritis have utilized AAV2 as a gene transfer vector, these results demonstrate that AAV2.5 may be better suited in these models, especially in models of local treatment where the FLS are the target cells for AAV transduction.


MFI 5.97

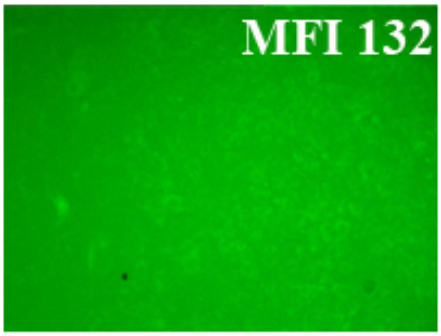
+ zLLL
MFI 81.6
MFI 551
AAV2
MFI 39.6
MFI 215
AAV2.5
MFI 8.18
MFI 47.2
AAV5

Figure 25: Ability of different serotypes of AAV to transduce human RA FLS.

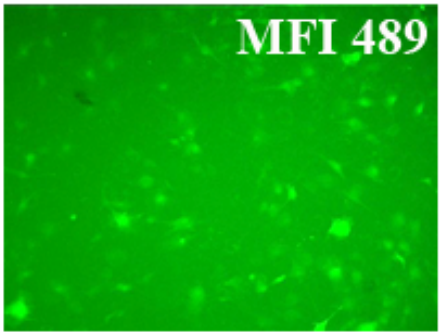
Cells were seeded at a density of $5x10^4$ cells per well in a 24 well tissue culture plate in RPMI media. The next day the cells were infected with $1x10^4$ particles per cell of dsAAV (EGFP) in a total volume of 0.4 ml. After a 6 hour infection, the cells were washed twice with PBS and fresh RPMI media was added. The following day the proteasome inhibitor zLLL was added to the indicated cells at a 20 μM concentration. zLLL was removed 24 hours later, the cells were washed twice with PBS, and 1 ml of fresh media was added. The next day the cells were trypsinized and analyzed by flow cytometry for EGFP expression. The results are representative of at least two separate experiments.

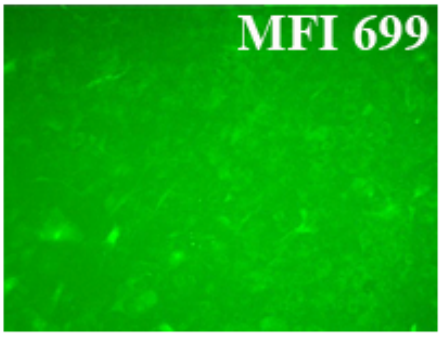


+ zLLL

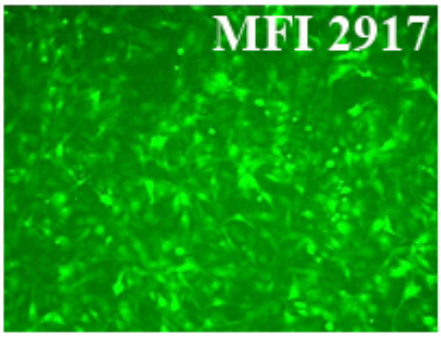


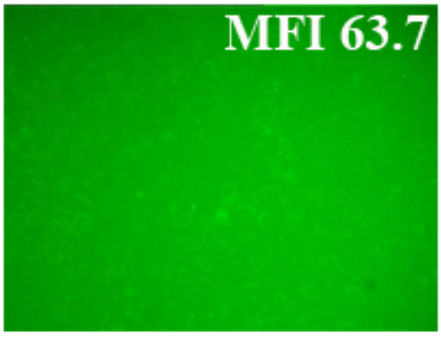
AAV2





AAV2.5





AAV5

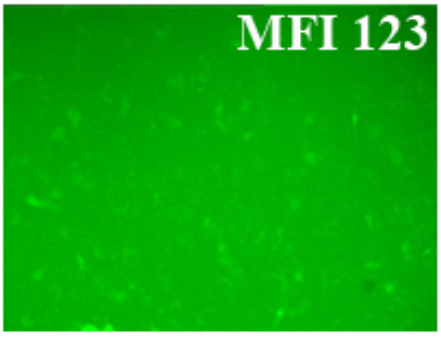
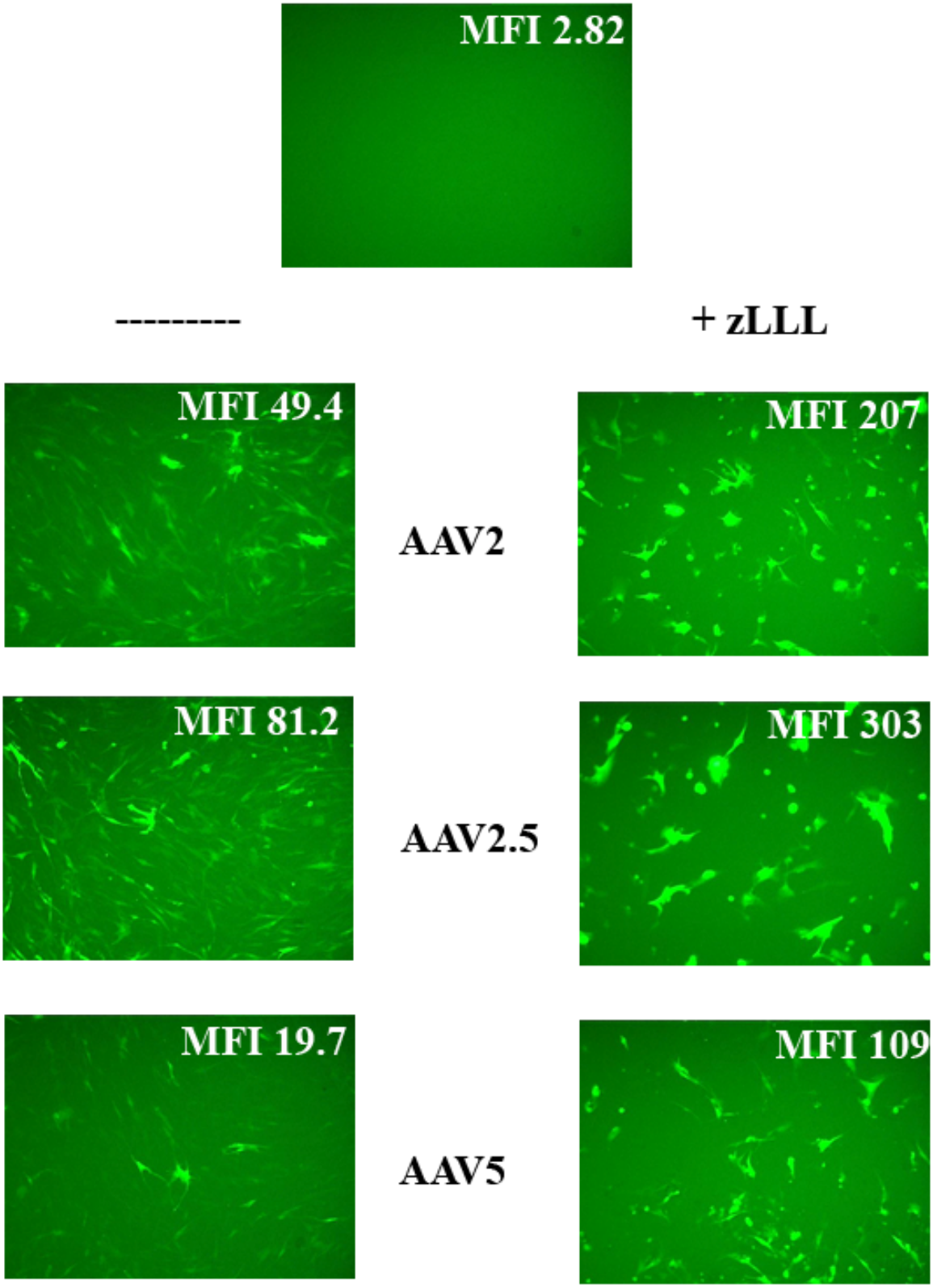


Figure 26: Ability of different serotypes of AAV to transduce mouse FLS.

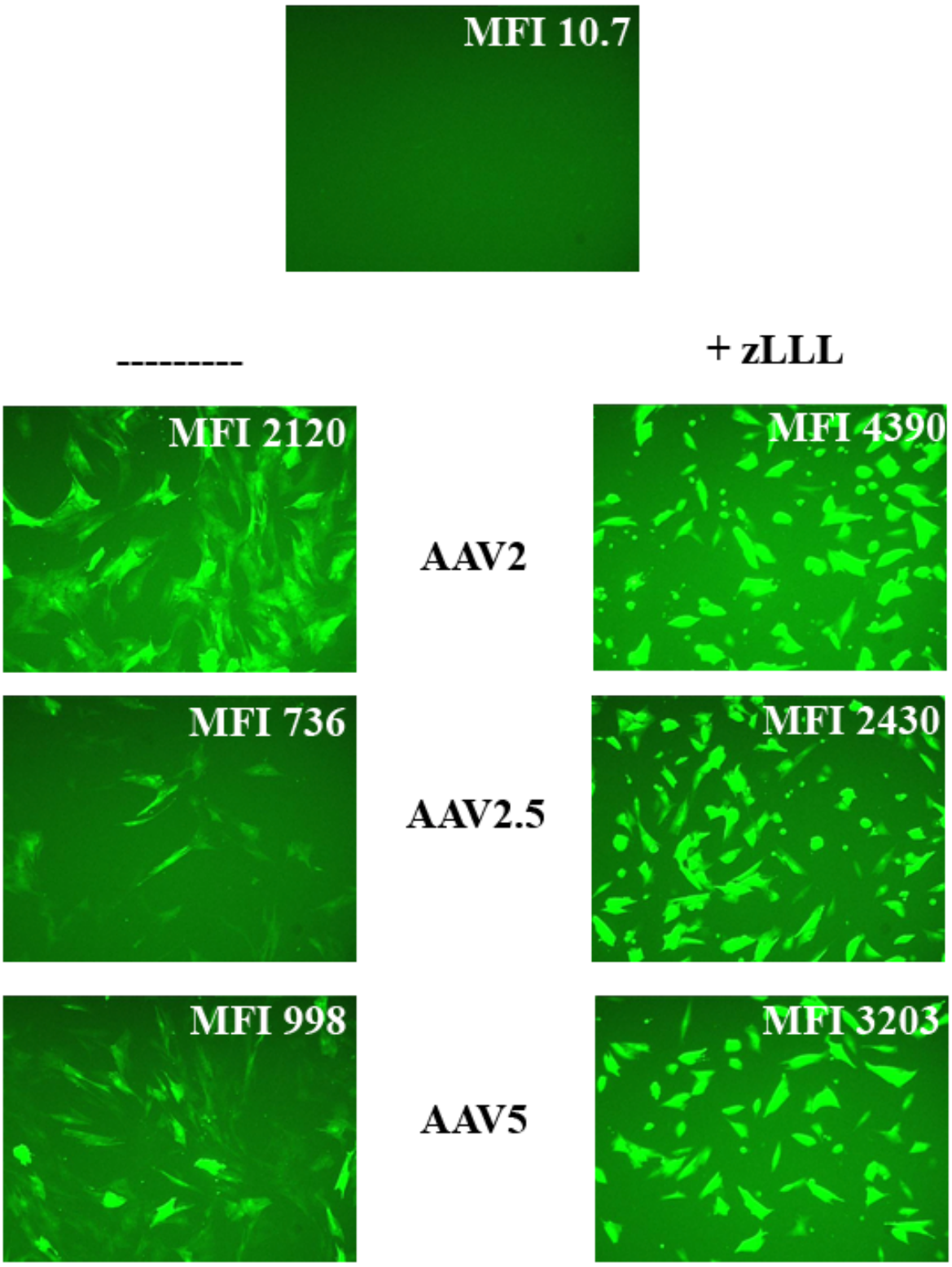
Cells were seeded at a density of $5x10^4$ cells per well in a 24 well tissue culture plate in RPMI media. The next day the cells were infected with $1x10^4$ particles per cell of dsAAV (EGFP) in a total volume of 0.4 ml. After a 6 hour infection, the cells were washed twice with PBS and fresh RPMI media was added. The following day the proteasome inhibitor zLLL was added to the indicated cells at a 20 μM concentration. zLLL was removed 24 hours later, the cells were washed twice with PBS, and 1 ml of fresh media was added. The next day the cells were trypsinized and analyzed by flow cytometry for EGFP expression. The results are representative of at least two separate experiments.


MFI 2.82

+ zLLL
MFI 49.4
AAV2
MFI 207
MFI 81.2
AAV2.5
MFI 303
MFI 19.7
AAV5
MFI 109

Figure 27: Ability of different serotypes of AAV to transduce rabbit FLS.

Cells were seeded at a density of $5x10^4$ cells per well in a 24 well tissue culture plate in RPMI media. The next day the cells were infected with $1x10^4$ particles per cell of dsAAV (EGFP) in a total volume of 0.4 ml. After a 6 hour infection, the cells were washed twice with PBS and fresh RPMI media was added. The following day the proteasome inhibitor zLLL was added to the indicated cells at a 20 μM concentration. zLLL was removed 24 hours later, the cells were washed twice with PBS, and 1 ml of fresh media was added. The next day the cells were trypsinized and analyzed by flow cytometry for EGFP expression. The results are representative of at least two separate experiments.


MFI 10.7

+ zLLL
MFI 2120
AAV2
MFI 4390
MFI 736
AAV2.5
MFI 2430
MFI 998
AAV5
MFI 3203

Figure 28: Ability of different serotypes of AAV to transduce horse FLS.

Cells were seeded at a density of $5x10^4$ cells per well in a 24 well tissue culture plate in RPMI media. The next day the cells were infected with $1x10^4$ particles per cell of dsAAV (EGFP) in a total volume of 0.4 ml. After a 6 hour infection, the cells were washed twice with PBS and fresh RPMI media was added. The following day the proteasome inhibitor zLLL was added to the indicated cells at a 20 μM concentration. zLLL was removed 24 hours later, the cells were washed twice with PBS, and 1 ml of fresh media was added. The next day the cells were trypsinized and analyzed by flow cytometry for EGFP expression. The results are representative of at least two separate experiments.

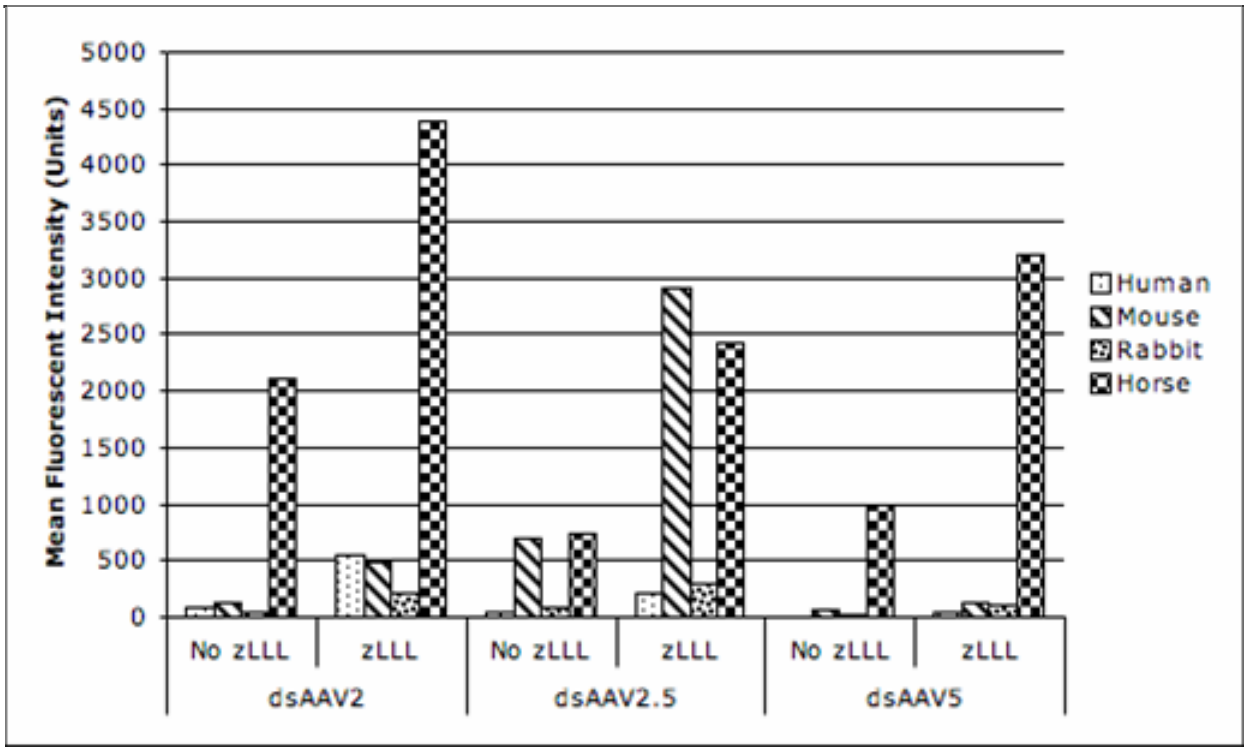


Figure 29: Summary of ability of different serotypes of dsAAV to infect fibroblast-like synoviocytes from different species.

4.4. Discussion

For the local treatment of arthritis to be successful using AAV vectors, it is important to determine which serotype is best suited for intra-articular transduction of FLS. The current AAV vectors in clinical trials for intra-articular delivery arthritis are derived from AAV2. In our studies in human FLS, all 3 serotypes tested provided very low levels of transgene expression, although out of AAV2, 2.5, and 5, AAV2 was best suited for transgene delivery to human RA FLS.

While therapeutic levels of a transgene may be attainable even at such low expression levels, our results demonstrate that administration of the proteasome inhibitor zLLL can increase transgene expression significantly. This suggests that intra-articular administration of zLLL at

the time of gene transfer or during flares of arthritis could increase therapeutic transgene expression dramatically, possibly leading to much quicker resolution of disease symptoms. Intra-articular injection of zLLL was able to protect rat chondrocytes from mono-iodoacetate cytotoxicity by induction of the heat shock protein Hsp70 (316).

Our results also highlight the dramatic differences in the ability of different serotypes of AAV to infect the same cell type from different species. There are two considerations in the design of a gene transfer study using AAV where the choice of which serotype to use is particularly important. If the primary goal is to show efficacy of delivering a particular transgene for the treatment of a disease, then choosing a serotype that provides the highest level of transgene expression in the model under study is of utmost importance. However, it is important to realize that this serotype may not necessarily be the best suited for future studies that may examine delivery of the same transgene to human tissues. If the primary goal of a study is to obtain the highest level of transgene expression possible in human tissue, several serotypes should be screened for their ability to infect that tissue. In addtion, other manipulations, either to the virus itself or to the infected cells, may improve transduction (discussed in chapter 1) and should be thoroughly examined before moving on to clinical trials. Clearly, for local models of mouse and rabbit arthritis using AAV, AAV2.5 provides the highest level of transgene expression. As discussed above, however, AAV2.5 would not be well suited for local treatment of human RA.

In our studies, horse FLS provided the highest level of transgene expression with all 3 serotypes of AAV, suggesting an equine model of arthritis would be ideal for examining the efficacy of intra-articular adiministration of AAV vectors. However, there is currently no *in vivo* model of equine arthritis, and the cost of maintaining such a model would likely be prohibitively

expensive. Alternatively, it appears that mouse models of intra-articular delivery of AAV should be performed with AAV2.5. This serotype will provide the highest level of transgene expression and will therefore likely yield the most conclusive results.

The proteasome inhibitor zLLL increased transgene expression from all 3 serotypes equally. The only difference between the AAV serotypes is the capsid of the virus. If the mechanism of action of zLLL is on the intracellular trafficking of AAV, this suggests that these three serotypes share the same pathway of infection, at least in this cell type. It is also possible that zLLL acts on a transcriptional or post-transcriptional level, as the viral genome encapsidated within all 3 viruses was identical. Second-strand sythesis is also not involved, as these vectors were self-complementary. Apical infection of polarized airway epithelia with both dsAAV2 and dsAAV5 in the presence or absence of the proteasome inhibitor LnLL gave similar results (231). This suggests that these viruses share an intra-cellular barrier to infection that can be overcome by inhibition of the proteasome.

5. Summary and Future Directions

Rheumatoid arthritis is a debilating disease affecting millions of people across the globe. RA is characterized by inflammation of the joint, with an overproliferation of the cells of the synovial lining, creating a pannus of tissue that invades the cartilage and destroys bone. Although there remains no cure for RA, there has been significant progress in the development of new therapies in recent years. The most successful of these therapies has been aimed at neutralizing the pro-inflammatory cytokines TNFα and IL-1β. These cytokines contribute to the pathogenesis of RA by perpetuating cartilage and bone destruction. Current treatments are often given systemically, which can lead to the development of significant side effects. There have been reports of re-activation of tuberulosis and the development of lymphoma in patients taking

anti-TNFα medications. Intra-articular administration of these drugs could prevent the development of systemic side effects, but the half-life of these agents is so short that the frequency of administration that would be required makes it unfeasible.

Gene therapy is increasingly becoming a viable treatment option for many diseases, including RA, and has the potential to overcome many of the limitations of more traditional treatment regimens. Intra-articular administration of gene transfer vectors would require few, if any, repeat administrations, thereby providing the benefits of local treatment without the drawback of systemic side effects. One of the most attractive vectors for gene transfer is AAV. It causes no known human pathology and has broad tissue tropism. It also has low immunogenicity and is associated with long-term gene expression. For RA in particular, the fibroblast-like synoviocytes lining the joint are being targeted for gene transfer because of the prominent role they play in the pathogenesis of RA. These cells are permissive for AAV transduction, but provide low levels of transgene expression. Our studies sought to find ways to improve AAV transduction of fibroblast-like synoviocytes, potentially making local gene transfer of AAV vectors to the joint a viable treatment option for RA.

We found that the inflammatory cytokines TNFα, IL-1β, and IL-6 were able to increase transgene expression in FLS in a PI3K and NFκB-dependent manner. Synovial fluid from RA patients, but not from controls, also increased AAV transgene expression in FLS, suggesting that the level of inflammatory cytokines present in the joint of RA patients is sufficient to increase expression from AAV vectors. The most striking result was that the inflammatory cytokines could regulate transgene expression in FLS. This indicates the local inflammatory environment of the arthritic joint can promote transgene expression. The therapeutic transgene can then act to decrease inflammation in the joint, at which time trangene expression would return to basal, low

levels. During active flares of arthritis, transgene expression would again be turned on until the inflammation was brought under control. This self-regualtory control of transgene expression makes AAV particularly appealing as a gene transfer vector for inflammatory diseases such as RA.

The mechanism by which inflammatory cytokines increase AAV transgene expression in in FLS is a little less clear. Both PI3K and NFκB appear to play a role. Confocal microscopy revealed that the inflammatory cytokines promote uncoating of the virus. PI3K activity is required for efficient trafficking to the nucleus. The literature suggests the virus is uncoated prior to or during nuclear translocation. This suggests that inhibition of PI3K may prevent cytokines from promoting uncoating of the virus. Further studies will have to be performed to determine if this is indeed the case. NFκB is a transcription factor and therefore likely plays a role at the transcriptional level. There are four consensus binding sites for NFκB on the CMV promoter and enhancer. When NFκB is inhibited, inflammatory cytokines are unable to increase AAV transgene expression. While this suggests that the inflammatory cytokines act in an NFκB-dependent manner, it is also possible that the cytokines act further upstream in the infectious process, prior to transcription (i.e. uncoating). Even if the cytokines promote uncoating and efficient trafficking of the virus to the nucleus, global inhibition of NFκB would still be expected to prevent efficient transcription from the viral genome, since the CMV promoter is thought to be primarily under control of NFκB. Testing the effect of NFκB inhibition on the ability of cytokines to increase transgene expression from a virus with a different promoter (i.e. a chicken beta actin promoter) may shed more light on this phenomenon.

Proteasome inhibition was found to increase transgene expression in FLS, also in a regulatory fashion. Nearly 80% of cellular proteins are degraded by the ubiquitin/proteasome

system. Therefore, proteasome inhibiton would be expected to effect many cellular processes. Despite this fact, proteasome inhibitors have demonstrated success in the treatment of multiple myeloma. In addition, it may be possible to inject the proteasome inhibitor intra-articularly, thereby limiting systemic side effects. In our experiments, the proteasome inhibitor zLLL appeared to increase AAV transgene expression by both transcriptional effects and effects on the intracellular localization of AAV viral DNA. The global cellular effects of proteasome inhibition make it difficult to determine which of these mechanisms may be playing a more significant role, and requires futher examination. Interestingly, treatment of infected cells with zLLL appears to lead to the accumulation of viral particles in aggresomes. How this could affect levels of transgene expression has yet to be determined. The effect of zLLL does not involve the transcription factor NFκB, as proteasome inhibition in cells with NFκB inhibited still resulted in increases in AAV-mediated transgene expression. This is in contrast to the effect of cytokines mentioned above, which was NFκB-dependent, indicating that cytokines and proteasome inhibitors increase AAV transgene expression by different mechanisms. The data gathered with regards to the mechanistic effects of cytokines and zLLL are summarized below in Table 3.

Table 3. Summary of mechanistic effects of cytokines and zLLL on AAV transgene expression.

	CYTOKINES	zLLL
Regulation?	+	+
NFκB-dependent?	+	-
PI3K-dependent?	+	+/-
mRNA levels?	+	+
Effect on intact particles	Loss of intact particles	Accumulation in "aggresomes"

To this point, the data suggest that cytokines and zLLL increase AAV transgene expression by separate mechanisms. The primary data that suggests this is the NFκB dependency of the effects of cytokines, while the zLLL effect is NFκB-independent. In addition, cytokines and zLLL have differing effects on the existence and location of intact particles within the cell. To determine if the location or amount of intact capsids within the cell is at all related to the increase in transgene expression, the effects of cytokines and zLLL should be tested at least 10 days after infection. At this time, trafficking within the cell would be presumed to be complete. In addition, there appears to be a general decrease in the number of intact capsids within the cell over time. If all the viral particles have been uncoated and cytokines and zLLL still exert effects on transgene expression (which we have observerved at these later time points), this would suggest that the primary effect is not due to the presence or absence of intact particles

and their location within the cell. From the data gathered to date I would expect that intact particles would no longer be visible within the cell at these later time points but we would still observe large increases in transgene expression.

Once the effect on intact particles has been ruled out as contributing to increases in transgene expression, the next step would be to determine the location of the viral DNA within the cell. This experiment could be done using *in situ* hybridization with a probe specific for the viral DNA, presumably to the viral ITR, promoter, or transgene (assuming it is a reporter gene and not a transgene that would be expected to also be present in the human genome, i.e. IL-10). This experiment would act to confirm our results that zLLL causes a shift of viral DNA from the peri-nuclear cytoplasm to the nucleus, with an eventual drifting back to the cytoplasm over time. If the effect we observed was an artifact of damage to the nuclear envelope caused by proteasome inhibition, it will be revealed in this experiment. In the same vain, we did not observe this effect upon addition of cytokines, and this experiment would confirm this as well. Co-staining with markers for intracellular compartments and/or the nuclear envelope should help to localize the viral DNA within the cell.

For the cytokine treated and untreated cells, I would hypothesize that the viral DNA would be located primarily in the nuclear compartment, with no changes in the quantity of viral DNA in the nucleus upon cytokine addition. This would suggest that the primary effect of cytokines is on transcription or a post-transcriptional step. For zLLL, I would hypothesize that we will confirm our data demonstrating a shift of viral DNA from the cytoplasm to the nucleus and back again. Proteasome inhibition no doubt is a warning signal to the cell that some type of damage is occurring. To prevent uncontrolled gene transcription, the cell may try to sequester DNA away from transcriptional machinery. For chromosomal DNA this might be done by

alterations to nucleosome and histone organization, thereby preventing transcription of some or all genes. For episomal elements like viral DNA, it is entirely possible that some mechanism exists to sequester the DNA just outside or within the nuclear membrane. If this is the case, it should be revealed by the *in situ* hybridization experiment. If *in situ* hybridization demonstrated no trafficking of viral DNA within the cell, this would suggest the effect is primarily on a transcriptional or post-transcriptional level. If this is the case, either transcription factors other than NFκB are involved or the effect is at the level of mRNA stability or post-translational control.

The cytokine effect appears to be much more likely to be due to effects on gene transcription, as the dependency on NFκB suggests. The same effect was also observed using a virus with the chicken beta actin promoter. However, the presence of the CMV enhancer in this construct still allows for NFκB to have an effect. If the NFκB sites in this virus were mutated and the cytokines still had an effect, this would suggest that the primary effect is either due to other transcription factors (mentioned above) or the viral ITRs. The role of the viral ITRs in this process is being actively pursued by our laboratory. If the viral ITRs play a role, we would expect a construct missing the ITRs to be unresponsive to inflammatory cytokines. I would hypothesize that the effect is dependent on the viral ITRs. Binding of factors to the ITRs may act alone or in concert with factors that bind to the promoter of a specific viral construct (i.e. CMV or CB actin). This could explain why the effect might be dependent on NFκB, but yet might still be observed with a virus using another promoter (i.e. CB actin). To dissect the role of ITRs in more detail, the signaling pathways activated within the cell could be examined with phospho-specific antibodies to molecules such as Erk and Jnk, for example, and electrophoretic

mobility shift assays could be performed using the viral ITRs as substrates to attempt to determine what factors may bind to this region.

Similarly, the role of PI3K in this process could be examined further. The fact that wortmannin blocks increases in transgene expression in response to cytokines several days after infection (when trafficking would be presumed to be complete) suggests that although PI3K activity is required for efficient trafficking of the virus to the nucleus, it may also have effects on transcription, translation, or post-transcriptional processing as well. Alternatively, PI3K activity may be required for the activation of production of a factor that may bind to the viral ITR, as discussed above.

Several serotypes of AAV were tested for their ability to infect FLS from four different species: human, mouse, horse, and rabbit. AAV2 was found to be most suitable for transduction of human and horse FLS, while AAV2.5 provided the highest transgene expression in mouse and rabbit. Proteasome inhibition was able to increase trangene expression in all cell types infected with all 3 AAV serotypes. This suggests that zLLL acts either on a step in viral trafficking that is shared between all 3 serotypes or acts on a transcriptional or post-transcriptional level.

Several animal models of arthritis exist, many of which are mouse models. The most widely used mouse model is collagen-induced arthritis (CIA). Several gene transfer studies examining intra-articular injection of AAV vectors have been performed, most of which use AAV2. Our results suggest that AAV2.5 will provide higher transduction in mouse FLS, thereby allowing a better evaluation of the therapeutic effects of the particular transgene being examined.

Overall, our data show that both inflammatory cytokines and proteasome inhibitors can regulate AAV-mediated trangene expression in FLS. Therefore, AAV may be ideally suited for

local gene transfer to the joint as a therapy for RA. These studies have potential implications in the future treatment of RA using AAV vectors.

APPENDIX A

Publications

Traister, RS, and Hirsch, R. "Gene Therapy for Arthritis," Immunologic Research, in press.

Traister, RS, Fabre, S, Wang, Z, Xiao, X, and Hirsch, R. "Inflammatory cytokine regulation of transgene expression in human fibroblast-like synoviocytes infected with adeno-associated virus," Arthritis and Rheumatism, Vol. 54, No. 7, July 2006. pp. 2119-2126.

Jennings, K., Miyamae, T., Traister, R., Marinov, A., Katakura, S., Sowders, D., Trapnell, B., Wilson, J.M., Gao, G., and Hirsch, R., 2004. "Proteasome inhibition enhances AAV-mediated transgene expression in human synoviocytes in vitro and in vivo," Molecular Therapy, Vol. 11, No. 4, April 2005. pp. 600-607.

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