

ALPHA-1 ANTITRYPSIN PROTEIN AND GENE THERAPIES FOR THE PREVENTION OF
RHEUMATOID ARTHRITIS IN MOUSE MODELS

By

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To my parents, Herbert and Ursula Grimstein

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Rheumatoid arthritis (RA) is an autoimmune disease characterized by immune cell infiltration, synovial hyperplasia, and progressive destruction of bone and cartilage, affecting about 1% of the population in the United States. Recent studies have suggested that RA results from autoimmune self destruction and imbalance between inflammatory and anti-inflammatory network.

Human alpha 1 antitrypsin (hAAT) is a multi-functional protein with anti-inflammatory and protective properties. We have previously shown that treatment of hAAT prevents type 1 diabetes development and protects islet cells from apoptosis in mouse models. However, the effect of hAAT on RA has not been tested.

The goal of this study is to determine the protective effect of hAAT protein therapy and gene therapy on the development of RA and autoantibody formation in collagen-induced arthritis (CIA) and pristane induced arthritis (PIA) mouse model.

First, hAAT was delivered either intraperitoneally as a human protein or with a recombinant adeno-associated virus vector expressing hAAT (rAAV8-CB-hAAT) in CIA model. Using these different delivery approaches, we examined the feasibility of hAAT as a therapeutic

agent for RA. We were able to show that both approaches successfully suppressed collagen II autoantibodies and delayed arthritis onset in DBA/1 mice.

We intended to further improve our therapeutic approach and delivered rAAV8-tet-on-hAAT vector expressing hAAT in combination with doxycycline. Interestingly the combination therapy was more effective in suppressing arthritis development than monotherapy of hAAT or doxycycline alone.

In addition, we tested the feasibility of hAAT therapy in pristane induced arthritis mouse model. Although the effect of hAAT on arthritis scores was not significant due to the variation of the model, hAAT and doxycycline combination therapy was able to suppress lupus related autoantibody anti-Su.

These results suggest that hAAT has therapeutic potential for the prevention of disease progression and the treatment of RA. The therapeutic effect of hAAT appears to be at least in part through the control of autoantibody development and can be further improved by combination with doxycycline.

CHAPTER 1 INTRODUCTION AND BACKGROUND

Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a systemic autoimmune disease, and affects about 0.5 to 1% of the population in the industrialized world (1). As the most frequent of all inflammatory joint diseases, it is characterized by chronic joint inflammation and synovial hyperplasia leading to bone and joint destruction. Insufficiently treated, RA leads to disability resulting in loss of working capacity, early retirement as well as premortal death and therefore has not only a great impact on quality of life for the individual but also on health care and socioeconomic systems (2, 3).

Pathogenesis and Treatment of Rheumatoid Arthritis

Although the exact causes or the initiating stimuli remain unclear, extensive research over the last decades have shown that multiple genetic as well as environmental factors interact and trigger the onset of RA (4, 5). Multiple cell types, associated with the immune system, enter the synovium via activated endothelial cells which express various adhesion molecules. Among those cells are dendritic cells, neutrophils, macrophages, fibroblasts, mast cells, natural killer (NK) cells, NKT-cells, T-cells as well as plasma cells. Especially dendritic cells express pattern recognition molecules such as Toll-like receptors, which can bind foreign antigens and self structures, become subsequently activated and act on cells of the adaptive immune system.

One of the key players in arthritis pathogenesis are T-cells. They recognize antigens which are associated with arthritis and are presented by professional antigen presenting cells (APC's) such as dendritic cells, macrophages or activated B-cells. The antigenic peptides are presented by class II MHC molecules, located on the surface of APC's. It has been shown that 80% of RA patients carry a so called shared epitope of the HLA-DRB1*04 cluster indicating a relationship

between disease susceptibility and certain antigenic peptides (6). These peptide-MHC complexes lead to stimulation and expansion of auto antigen specific T-cells in joints or lymph nodes, or both. Besides T-cell receptor engagement with peptide-MHC complexes, full T-cell activation requires a co-stimulatory signal which is mediated through CD80 and CD86, receptors located on the APC surface and the T-cell receptor CD28 (7).

To avoid over stimulation, T-cells also express cytotoxic T-lymphocyte antigen 4 (CTLA-4) which binds with higher affinity than CD28 to APC receptors CD80/CD86 and conveys inhibitory signals. Abatacept is a recombinant protein of CTLA-4 and blocks CD80 or CD86 mediated co-stimulation and activation of T-cells and is therefore used therapeutically in rheumatoid arthritis.

T-cells in the synovium usually belong to Th1 subset and their up regulation is associated with the expression of various cytokines such as interleukin 2 (IL-2) and interferon γ (INF- γ). Once stimulated, these T-cell are able to induce macrophages, B-cells, fibroblasts, and osteoclasts (8, 9). B-lymphocytes express various surface receptors such as their antigen receptors, immunoglobulins, and differentiation antigens, such as CD20 and CD22. They are able to differentiate into plasma cells which secrete antibodies, including autoantibodies to IgG (rheumatoid factor), citrullinated peptides such as vementin, fibrinogen, cyclic citrullinated peptide or collagen II (10). These autoantibodies are able to form immune complexes that stimulate proinflammatory cytokine production via complement and Fc receptor activation (11). It has been shown in different studies that presence of autoantibodies is associated with severe rheumatoid arthritis (12, 13). Since B-cells also can serve as APC's, they are also able to activate T-cells and contribute to perpetuation of the autoimmune response (14, 15). The survival and activation of B-cells, including expression of different immunoglobulin isotypes, are mediated

and maintained by the interaction with T-cells and co-stimulation. Rituximab, a monoclonal antibody against CD20 is another recently licensed agent that targets and depletes B cells.

In addition to immune cell infiltration into the inflamed synovium we also observe neovascularisation and a large increase in fibroblasts like synoviocytes, which are highly activated and produce cytokines and inflammatory mediators such as prostaglandins and matrix metalloproteinases (MMP) (16, 17). Fibroblasts like synoviocytes secrete matrix metalloproteinases (MMP's) directly into the synovial fluid and also invade the surrounding tissues, leading to destruction of cartilage and bone (18, 19).

Many cytokines are secreted by activated cell populations in the synovium (20). Proinflammatory cytokines, such as TNF- α , IL-1 β or IL-6 secreted by activated macrophages and fibroblasts have shown to be important contributors for disease pathogenesis. These cytokines can therefore be therapeutic targets. TNF- α inhibitors such as Infliximab, Adalimumab and Etanercept, IL-1 β receptor antagonist Anakinra, or humanized IL-6 receptor Tocilizumab have been approved and tested in recent years for the treatment of rheumatoid arthritis in humans (21). It is interesting to note that when monotherapies fail, the therapeutic effect of these biologics can be further enhanced by combination therapies with methotrexate, which inhibits lymphocyte proliferation and is a cornerstone drug in RA treatment. (3, 22-25).

The use of biologics and disease modifying drugs (DMARD) such as methotrexate, leflonomide, sulfasalazin and hydroxychloroquine in monotherapy as well as in combination therapy have shown improved treatment success in rheumatoid arthritis patients (26). However, despite promising effects regarding effective suppression of disease development, current therapies are accompanied by side effects and lack of efficiency in some patients. Increased risk of liver damage, infections, malignancies (anti-cytokine therapy) and teratogenesis have been

reported after treatment in RA patients (27-29). Three quarters of patients treated with biological agents do not reach American College of Rheumatology 70% improvement score (21). Therefore there is still need to optimize current therapies as well as developing new drugs and treatment strategies.

Animal Models for Rheumatoid Arthritis

There are a number of adequate animal models for rheumatoid arthritis which are commonly used in arthritis research. Their heterogeneity in disease stimulating factors and presentation of disease progression resemble the heterogeneity of clinical symptoms among patients. They are used to simulate pathogenesis and progression of the human disease and may predict efficiency of therapeutic agents in humans. Most models are easy to perform, show reasonable duration of active disease state and reproducibility of data.

In many studies arthritis models of rats or mice are used and arthritis development is induced in different ways. Streptococcal cell wall (SCW) induced arthritis is the best characterized arthritis animal model in rats. A single injection of peptidoglycan polysaccharide leads to chronic, severe and erosive arthritis development within days. The model is characterized by a high incidence of 95% and females are more effected then male, similar to what is observed in humans (30). Interestingly SCW induced arthritis in mice displays a short active state of disease of less then 7 days and absence of the chronic phase (31). The SCW induced arthritis model as well as collagen induced arthritis, which is described below, develop arthritis due to administration of foreign antigens following an antibody dependent immune response targeting the joint tissue. It also has been shown that injection of anti-collagen antibodies cocktail or immune complexes carrying joint unrelated proteins are able to induce severe and chronic arthritis in susceptible mouse strains. Intravenous injection of heat-inactivated polyclonal rabbit anti-lysozyme serum, followed by an injection with poly-lysine-

coupled lysozyme leads to immune complex formation and subsequent inflammatory arthritis (32).

In contrast to the described models above, some mouse strains develop arthritis without prior administration of external antigens, adjuvant or antibodies. Due to their genetic background K/BxN or NZB/NZW mice develop inflammatory and progressive arthritis spontaneously. K/BxN mice are characterized by a mutation in a T-cell receptor and therefore develop antibodies to ubiquitous glucose-6-phosphate isomerase. Formation of immune complexes including these antibodies is responsible for arthritis development in these mice at the age of 3 weeks (33). NZB/NZW mice also spontaneously develop inflammatory arthritis which is characterized by generation of IgG and IgM rheumatoid factor, similar to human disease. As in pristane induced arthritis mouse model which is reviewed in more detail below, these mice also develop lupus related autoantibodies and are therefore used as a model for systemic lupus erythematosus(34).

Other genetic models include gene modifications in TNF- α gene or knockout of IL-1 receptor antagonist leading to inflammatory arthritis and emphasizing the importance of these two cytokines in disease pathogenesis(34).

For our studies, we employ collagen induced arthritis mouse model which is commonly used, well described and displays reproducibility, high incidence and convenient induction. The pristane model we used displays slightly different disease conditions because it is different in terms of time of disease onset and duration of disease progression.

Collagen Induced Arthritis (CIA)

Collagen induced arthritis (CIA) has been proven to be an excellent animal model for rheumatoid arthritis since it shares several characteristics with the human disease. Among those are genetic, pathological and immunological features. CIA has been developed in a number of

species including mice, rats and monkeys. Although initially developed in rats, CIA is now the most widely used mouse model for studying autoimmune arthritis pathogenesis. After immunization with type II collagen these animals develop progressive and inflammatory arthritis within days as well as high serum levels of auto-reactive antibodies against collagen II. These auto-antibodies are involved in triggering inflammatory pathways via the activation of the complement cascade (35). The susceptibility is clearly associated with the expression of specific class II MHC genes and it has been shown that both T and B cells responses are required to establish the pathogenesis.

Pristane Induced Arthritis (PIA)

Pristane (2,6,10,14-tetramethylpentadecane) is a mineral oil which induces a chronic inflammatory arthritis in mice after intraperitoneal injection (36). Like other models of arthritis such as adjuvant-induced arthritis, collagen-induced arthritis, and streptococcal cell wall-induced arthritis, PIA resembles the joint inflammation and destruction seen in human RA. The main histological features are synovial hyperplasia, inflammatory cell infiltrate, bone erosions, cartilage erosions, and pannus-like formation. PIA demonstrates a prolonged, delayed clinical time course of joint inflammation which can start between 60 days and 180 days after pristane administration (36, 37). The condition is normally permanent, thus providing extended periods of disease for experimental investigation.

Like CIA, pristane induced arthritis is an inflammatory joint disease accompanied by hypergammaglobulinemia with a profile of antibodies against HSP and collagen II as well as the presence of rheumatoid factor. In addition to that, susceptible mice also have been shown to develop lupus specific autoantibodies against nuclear proteins (anti-Su, anti-RNP, anti-Sm, anti-DNA, anti-P).

The precise mechanism underlying PIA remains unclear, but it has been shown that pristane facilitates autoimmune responses via immune activation in response to antigens found on prevalent microbes in the environment (38, 39). Therefore, housing mice in a pathogen-free environment precludes development of PIA, whereas returning such mice to normal environment reestablishes their susceptibility.

Gene therapy

In recent years, gene therapy has become a promising approach to mediate expression of therapeutic proteins in target tissues. Viral vectors such as lentivirus, retrovirus, adenovirus and adeno-associated virus or non-viral vectors such as liposomes or plasmid DNA are used for gene delivery (40, 41). Since the first clinical study using gene transfer was conducted by Rosenberg and his colleagues in 1990, the field has rapidly grown moving from preclinical studies to clinical studies for many diseases. Due to constant improvements of gene therapy vectors in terms of efficiency and safety, we are now able to ensure long term gene expression resulting in high levels of therapeutic proteins in serum and various tissues.

Several clinical gene therapy trials have been conducted or are currently in progress. They target diseases such as cystic fibrosis, alpha-1 antitrypsin deficiency, muscular dystrophy, Parkinson disease, cancer, hemophilia, Alzheimer's disease and severe combined immunodeficiency (42, 43).

Gene Therapy for Rheumatoid Arthritis

Gene therapy is a promising therapeutic approach for RA treatment since it overcomes some drawbacks of currently applied protein therapies. It resembles an effective, inexpensive and continual delivery system in which regular injections of protein is not necessary. The most prominent viral vectors used in arthritis therapy include retroviruses, lentiviruses, adenoviruses, herpes simplex viruses as well as adeno-associated viruses (44).

Several studies conducted in animal models showed that gene therapy delivering IL-1 receptor antagonist (IL-1RA), IL-18 binding protein, or TNF- α receptor variants effectively reduced collagen induced arthritis development in mouse model (45-50). Besides targeting cytokines, other studies using gene therapy showed that delivering transgenes which target the degradation of extracellular matrix has significant effect on suppression of arthritis development in rodents. Delivering tissue inhibitor of matrix metalloproteinases such as TIMP1, TIMP3 and TIMP4 or ribozymes targeting MMP-1 and cathepsin L mRNA have shown promising results using adenoviruses, retroviruses or electroporation (51-54). In addition anti-angiogenic gene therapy employing adenovirus or adeno associated virus to deliver proteins with anti-angiogenic properties such as angiostatin, human kallistatin, TSP-1 or soluble sFlt-1 showed promising potential to reduce arthritis development in mice and rats (55-57). Targeting apoptosis using adenovirus to deliver Fas-ligand or TRIAL or delivering anti-inflammatory cytokines such as IL-4 and IL-10 also showed that there is indeed a wide range of potential target molecules which are currently used for anti-arthritic gene therapy (58-65).

Less than 10 gene therapy clinical trials have been initiated for the treatment of rheumatoid arthritis to date (66). For most of them results have not been published yet. In one clinical trial, an IL-1RA transgene has been ex vivo transduced by an retrovirus and a dose escalation study in 9 volunteers confirmed feasibility and safety as well as evidence supporting symptomatic relief (67, 68). Another study, sponsored by ISIS pharmaceuticals, was designed to deliver antisense oligonucleotides, reducing the production of TNF by blocking mRNA. The study aimed to assess safety and efficacy in a randomized double blind, placebo control design. No results have been published yet, but a press release on the companies' website suggests positive disease response in patients receiving the highest dose.

The most recent human clinical arthritis trial performed by Targeted Genetics, in which i.a. delivery of a TNF- α receptor was mediated by adeno-associated virus serotype 2 (rAAV2) also revealed promising results. The phase 1/ 2 study showed improvement of joint symptoms, function and pain. Although one patient died after receiving a second injection of AAV2 and the FDA put the trial temporarily on hold, it was confirmed that AAV gene therapy was not related to the incidence and a severe histoplasmosis infection most likely caused the patient death (69).

Using gene delivery vehicles to specifically target cells and mediators involved in disease development reveal a promising potential for effective arthritis therapy. Further research has to be conducted to elucidate safety and long term efficiency of this novel treatment approaches.

Adeno-Associated Virus (AAV) Mediated Gene Delivery

Adeno-associated viruses (AAV) are small, non enveloped single- stranded parvoviruses that depend on a viral helper, such as adenovirus or herpes simplex virus, to facilitate productive infection and replication. In the absence of a helper virus, AAV establishes a latent infection within the cell, resulting in long-term expression in vivo, either by site-specific integration into the host genome or by persisting in episomal forms. AAV has never been associated with any human disease and recombinant AAV vectors have never led to significant inflammatory responses or toxicity in animal models and human clinical trials (70). In this sense, rAAV is the safest of the currently used gene therapy vectors. Because of its propensity to establish latency and because it has not been implicated as a pathogen, AAV has been of considerable interest as a potential vector for human gene therapy (71). In addition rAAV has mediated long- term transgene expression in a wide variety of tissues, including muscle (72-76), lung (77), liver (76, 78-80), brain (81) and eye (82). Therefore rAAV vectors appear to have significant advantages over other commonly used viral vectors.

So far, 12 primate rAAV serotypes have been isolated and cloned. Serotype 2 (AAV2) is the best-characterized and has been predominantly used in gene therapy studies. Apparailly et al. recently demonstrated that rAAV5 is the preferred serotype for local gene delivery to joints in RA since it efficiently transduces synovial cells (83). rAAV 8 has been shown to be able to very successfully transduce murine liver (84) and muscle cells (85) in addition to a wide range of other tissues. The different tropism of AAV serotypes provides opportunities to optimize the transduction efficiency in different target cells. It is therefore the vector of choice when systemic gene delivery is desired.

Alpha-1 Antitrypsin (AAT)

Human alpha-1 antitrypsin (hAAT) is a 52 kDa serum glycoprotein, synthesized primarily in the liver. It is also expressed in other types of cells including neutrophils, monocytes, macrophages, alveolar macrophages, intestinal epithelial cells, carcinoma cells and the cornea (86-89). The normal serum level of hAAT is 1-2 mg/ml. During inflammation, hAAT level, as an acute phase reactant, can increase 3-4 folds. This makes it an ideal candidate protein as potential anti-inflammatory agent since it could be used in a wide therapeutic range without toxic side effects. Increasing evidence indicates that hAAT is immunoregulatory, anti-inflammatory and may be used for the treatment of RA. It inhibits neutrophil elastase and proteinase 3 with high efficiency, as well as cathepsin G, thrombin, trypsin and chymotrypsin with lower efficiency (90). Most of these proteases target receptor proteins, involved in proinflammatory cytokine expression and cell signaling (91). It also has been reported that neutrophil elastase inhibitors reduce incidence as well as severity of collagen-induced arthritis (CIA) in both rats and mice (92). Human AAT is able to completely eliminate the acute inflammatory infiltration and connective tissue breakdown in the lung in a cigarette smoke induced emphysema mouse model (93). It also inhibits LPS stimulated release of TNF- α and IL-1 β , and enhances the

production of anti-inflammatory cytokine IL-10 (94-96). Human AAT significantly protects against the lethality induced by TNF- α or endotoxin in mice (97). It can also induce expression of IL1-Ra in human PBMC's (98) and reduces ischemia-induced apoptosis and inflammation (99).

Doxycycline

Doxycycline, commonly used as an anti-microbial agent, has also shown to hold tissue protective properties. It is able to inhibit bone and cartilage breakdown in an animal model of osteoarthritis, and it also inhibits matrix metalloproteinases specifically the collagenases MMP8 and MMP13 (100, 101). Both collagenases are upregulated in RA patients and contribute to disease development of rheumatoid arthritis (102-104). Doxycycline also increases RNA degradation of nitric oxide synthase which usually increases cartilage breakdown and stimulates MMP expression in synovial cells (105). Furthermore, doxycycline also possesses anti-inflammatory effects. It induces FAS/FAS-Ligand mediated apoptosis in T-lymphocytes (106), and inhibits staphylococcal exotoxin stimulated T-cell proliferation and proinflammatory cytokine/chemokine production in human peripheral blood mononuclear cells (107).

Recently it has been shown, that combination therapy using doxycycline and methotrexate showed a significant American College of Rheumatology 50% improvement (ACR50) response in early seropositive rheumatoid arthritis patients when compared to methotrexate monotherapy (108). This study revealed promising potential for the use of doxycycline in combination therapies with other antiarthritic drugs.

Study Overview

The ultimate goal of the research presented in this dissertation is to test the feasibility of AAT as a therapy for rheumatoid arthritis. In recent years new developments have been made concerning RA pathogenesis and it has been shown, that reduction of inflammation and cartilage

destruction can be effectively achieved through targeting proinflammatory cytokines such as TNF- α and IL1- β .

Anti- TNF- α and anti-IL1- β cytokine therapy is now widely used for RA treatment and we believe that AAT with its anti-inflammatory and immune modulatory properties is effective in suppressing arthritis development by reducing joint inflammation. It may also avoid various side effects which are commonly seen in standard RA therapy.

Chapter 2 describes the experimental procedures used. Chapter 3 covers studies which were performed to test the feasibility of AAT to suppress arthritis development. We initially performed a protein therapy study to determine whether regular injection of commercially available hAAT protein (prolastin®) is able to reduce arthritis development in collagen induced arthritis mouse model. In addition we performed AAV mediated hAAT gene therapy in CIA model. Delivering hAAT in two different ways by employing protein as well as gene therapy intended to reveal the general potential effect of hAAT on RA development. Furthermore we also aimed to display a possible mechanism describing how AAT suppresses arthritis development. Therefore we determined levels of common RA biomarkers such as antibodies to collagen II in mouse serum and also tested hAAT effect on T-cell proliferation.

In Chapter 4 we specify the development of a combination therapy in CIA mouse model using hAAT and doxycycline. This approach has reference to common RA treatment practice since most therapies employed today are combination therapies of commonly used anti-arthritic drugs. Drug combination showed improved treatment effects in many RA patients.

The experimental approach described in Chapter 5 will further reveal the potential of hAAT on suppressing arthritis development and reducing autoantibodies. By using pristane induced arthritis mouse model we further widen the experimental setup in which anti-arthritic

properties of hAAT are tested. In addition we also intend to test the feasibility of the combination therapy using hAAT and doxycycline in this model and its potential to reduce autoantibodies.

Chapter 7 summarizes major findings and emphasizes future directions of the projects described.

CHAPTER 2 MATERIALS AND METHODS

Recombinant Adeno Associated Virus (rAAV) Vector Production

The rAAV-CB-hAAT vector construct was produced and packaged as previously described (82). Briefly, this vector carries hAAT cDNA driven by the cytomegalovirus (CMV) enhancer and chicken β -actin promoter and contains AAV2 inverted terminal repeats (ITRs) (Figure 2-1). The rAAV-tet-on-hAAT vector contains a bidirectional promoter with a tetracycline response elements (TRE) flanked by two mini-CMV promoters controlling hAAT and reverse transcriptional transactivator (rtTA) genes. In the presence of doxycycline, rtTA will bind to the TRE and activate hAAT and rtTA genes. The rtTA gene serves as a positive regulatory system for hAAT gene transcription (Figure 2-2). The respective plasmids were packaged into AAV serotype 8 capsid by cotransfection of vector plasmid and helper plasmid (XYZ8) into 293 cells. rAAV8-CB-hAAT and rAAV8-tet-on-hAAT vectors were purified by iodixanol gradient centrifugation followed by anion-exchange chromatography. The physical particle titers of vector preparations were assessed by dot blot analysis.

Human AAT Protein, Doxycycline and rAAV Vector Administration

For hAAT protein therapy studies, DBA/1 mice were intraperitoneally (IP) injected with 0.5 mg of hAAT in 100 μ l saline (Prolastin®, Bayer Corp., Elkhart, IN). The control group received saline injection. The injections were performed twice per week, starting from 6 days before the first bovine type II collagen (bCII) immunization.

For hAAT gene therapy studies, DBA/1 mice were IP injected with rAAV8-CB-hAAT vector (2×10^{11} particles/mouse) or saline two weeks before the first bCII immunization.

The combination therapy study consisted of four treatment groups, receiving a single IP injection containing rAAV8-tet-on-hAAT vector (1×10^{11} particles/mouse) or saline four weeks

before the first CII immunization. Some groups also received doxycycline during the study. Doxycycline was administered with food (ad libitum, 200mg/kg food). The groups represent hAAT and doxycycline combination therapy, doxycycline monotherapy, hAAT monotherapy and saline control group.

For studies using pristane induced arthritis model, cohorts of DBA/1 mice were IP injected with rAAV8-cb-hAAT (2×10^{11} particles/mouse) or saline. Some of the vector or saline injected mice also received doxycycline-containing food (ad libitum, 200mg/kg food). Therefore, there are 4 groups of mice: hAAT plus doxycycline, hAAT, doxycycline and saline group. Three weeks after vector injection, all animals received a single pristane injection to induce arthritis and lupus development.

Animals

Housing: DBA/1 mice were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN), housed in a specific pathogen-free room and handled as approved by the University of Florida Institutional Animal Care and Use Committee.

Collagen induced arthritis (CIA) mouse model: For induction of arthritis, bovine type II collagen (bCII, Chondrex LLC, Redmond, WA) was dissolved in 0.05N acetic acid at a concentration of 2mg/ml by stirring overnight at 4°C and emulsified with an equal volume of Complete Freund's Adjuvant (CFA, Chondrex LLC, Redmond, WA) or incomplete Freund's Adjuvant (IFA, Difco, Detroit, MI). Six week-old male DBA/1 mice were first immunized by subcutaneous injection at the base of the tail with 0.1ml of emulsion containing 100 µg of bovine type II collagen. Three weeks after priming (day 21), the mice were boosted with 0.1 ml of bovine collagen II (100µg) emulsified in equal volume of incomplete Freund's Adjuvant (IFA, Difco, Detroit, MI).

Pristane-induced arthritis mouse model: For pristane induced arthritis study, twelve week-old female DBA/1 mice received a single intraperitoneal injection of 0.5ml pristane (Aldrich Chemical Co., St Louis, MO) to induce arthritis and lupus development.

Evaluation of arthritis and lupus development: For assessment of arthritis, all mice were regularly monitored by the same person blinded to the treatment group and evaluated for incidence of arthritis and clinical score. An arthritis score system ranging from stage 0 – 4 was used (Figure 3-2): 0: no swelling or redness; 1: detectable arthritis with erythema; 2: significant swelling and redness; 3: severe swelling and redness from joint to digit; 4: joint stiffness or deformity with ankylosis (109). The clinical score was expressed as the average cumulative value of all four paws with a maximum score per animal of 16. Severe arthritis was defined as arthritis score >3 for the purpose of comparing data between groups. Serum was collected every 2 to 4 weeks for determination of hAAT and antibodies in serum. At 34 weeks after pristane injection, proteinuria was determined in urine using albutix (Bayer, Elkhart, IN), where 0 = absent, 1+ ≤ 30 (mild), 2+ = 100 (moderate), 3+ = 300(severe), and 4+ = 2000 mg/dl (very severe). All animals were sacrificed at the end of the respective study.

ELISA for the Detection of hAAT and Antibodies

Detection of hAAT and anti-hAAT antibodies in mouse serum was performed by standard ELISA as previously described (110). Purified hAAT was used as standard for detection of hAAT protein in serum (Athens Research & Technology, Athens, GA). High titer antibody sera were used as standard for determination of respective antibody levels.

Anti-type II collagen antibodies in mouse serum were also detected by a standard ELISA. Briefly, microtiter plates (Immulon 4, Dynex Technologies, Chantilly, VA) were coated with bCII or mCII (0.5 µg/well, Chondrex LLC, Redmond, WA) in Voller's buffer overnight at 4°C. After blocking with 3% bovine serum albumin, diluted serum samples were added and incubated

at room temperature for 2 h. HRP-conjugated goat anti-mouse IgG antibodies (1:1,000 dilution, Sigma, St. Louis, MO), goat anti-mouse IgG1 antibodies (1:1,500 dilution, Roche, Indianapolis, IN) or goat anti-mouse IgG2a antibodies (1:1,500 dilution, Roche, Indianapolis, IN) were incubated for 1 h at RT. The plates were washed with PBS-Tween 20 between reactions. After adding the substrate (o-phenylenediamine, Sigma, St Louis, MO) plates were read at 490 nm on an MRX microplate reader (Dynex Technologies, Chantilly, VA). Optical densities were converted into units based on a standard curve generated with high titer sera from DBA/1 mice immunized with bCII.

Anti-chromatin antibodies were detected in mouse serum (at a 1:500 dilution) using an ELISA with chicken erythrocyte chromatin, as described previously (111). In brief, microtiter plates (Immulon 4, Dynex Technologies, Chantilly, VA) were coated with 5µg/ml chicken erythrocyte chromatin in borate buffered saline overnight at 4°C. Sera were tested at a 1:500 dilution, followed by alkaline phosphatase conjugated goat anti-mouse IgG (1:1,000 dilution, SouthernBiotech, Birmingham, AL) and p-nitrophenyl phosphate substrate (Sigma, St Louis, MO).

Antigen-capture ELISAs were performed to detect anti-nRNP/Sm, anti-ribosomal P and anti-Su in the mouse serum (at a 1:500 dilution) as described previously (111, 112). Briefly, microtiter plates (Immulon 4, Dynex Technologies, Chantilly, VA) were coated with 1µg P peptide or 50µl of cell lysate from K592 cells (for anti-Su and anti-nRNP/Sm) overnight at 4°C. Sera were tested at 1:500 dilution followed by alkaline phosphatase conjugated goat anti-mouse IgG (1:1,000 dilution, SouthernBiotech, Birmingham, AL) and p-nitrophenyl phosphate substrate (Sigma, St Louis, MO).

Levels of anti-dsDNA and anti-single-stranded DNA (anti-ssDNA) antibodies were determined in the sera using S1 nuclease-treated calf thymus DNA as antigen (heat denatured for ssDNA) (111). In brief, microtiter plates (Immulon 4, Dynex Technologies, Chantilly, VA) were coated with 3µg/ml DNA in Pierce Reacti-Bind DNA coating solution (Pierce Biotechnology Inc. Rockford, IL) overnight at 4°C. Sera were tested at a 1:500 dilution, followed by alkaline phosphatase conjugated goat anti-mouse IgG (1:1,000 dilution, SouthernBiotech, Birmingham, AL) and p-nitrophenyl phosphate substrate (Sigma, St Louis, MO).

Total immunoglobulin levels were measured by ELISA as described previously (113). Briefly, 96-well microtiter plates (Immulon 4, Dynex Technologies, Chantilly, VA) were coated at 4°C overnight with 50 µl/well of goat anti-mouse kappa/lambda light chain antibodies (9:1 ratio, Southern Biotechnology, Birmingham, AL). Murine sera were tested at 1:200,000 dilution followed by a 1:1000 dilution of alkaline phosphatase-labeled goat anti-mouse antibodies specific for IgG1, IgG2a, IgG2b, IgG3 or IgM (Southern Biotechnology Birmingham, AL) and p-nitrophenyl phosphate substrate (Sigma, St Louis, MO).

Cell Proliferation Assay

To test the effect of hAAT protein administration on splenocyte proliferation, spleens from treatment and control group were harvested. Splenocytes were isolated and cultured in triplicates (4×10^5 cells/well, in 96-well plate) in serum free X-VIVO medium (Cambrex, Walkersville, MD) in the presence or absence of ConA (0.5 µg/ml, Sigma, St.Louis, MO). After culturing for 3 days, 1µCi/well of [³H] TdR was added. Cells were cultured for additional 18h and [³H] TdR uptake was measured using a β- scintillation counter.

Histological Assessment

For the analysis of acute arthritis in CIA model, mice were anesthetized and sacrificed by cervical dislocation. The two hind limbs of mice in treatment or control group were removed.

Specimens were fixed in formalin and decalcified in RDO solution (Apex, Aurora, IL) for 10-20 min depending on tissue size and then checked manually for pliability. Joint tissues were cut into 4 µm thick sections and stained with hematoxylin and eosin according to standard methods.

Histological evaluation was performed by two independent and blinded pathologists. Infiltration of immune cells, hyperplasia, pannus formation and bone deformation was determined for each paw using an evaluation scale ranging from 0-4 according to severity of histopathological changes (0: normal, 1: mild, 2: moderate, 3: severe, 4: very severe). The score was then averaged for each treatment group.

Immunohistochemistry

In order to detect transgene (hAAT) expression from rAAV8-cb-hAAT vector, saline and vector injected animals were sacrificed. From each animal, liver was harvested, fixed in 10% neutral-buffered formalin and submitted for routine paraffin processing. Formalin-fixed paraffin-embedded tissue sections (4µm) were sequentially deparaffinized, rehydrated, and blocked for endogenous peroxidase activity. Following antigen retrieval in Target Retrieval solution (Dako-Cytomation, Carpinteria, CA), sections were blocked with serum and incubated with either rabbit anti-hAAT (1:100; Research Diagnostic Institute, Flanders, NJ) or normal rabbit serum as a negative control. Antibody binding was detected using the Mach2 Polymer (Biocare, Concord, CA) and DAB+ (Biocare, Concord, CA). Slides were counterstained using hematoxylin (Vector Labs, Burlingame, CA) and mounted. Digital images were captured using a Zeiss Axioskop equipped with an Axiocam camera. Camera exposure settings were constant for all images.

Cell Culture

Mouse embryo fibroblasts cells (NIH/3T3) were purchased from American Type Culture Collection (Rockville, MD) and maintained in DMEM/10% Bovine Calf Serum (BCS) culture

medium (DMEM containing 10% heat-inactivated BCS, 100 units/ml of penicillin, 100 µg/ml streptomycin) at 37°C in a 5% CO₂ incubator.

For measuring IL-6 release, cells were seeded at 1×10^5 /ml in 12 well plates. After 48 h at 37°C, the culture medium was replaced with 0.5 ml of fresh, DMEM/10% BCS culture medium and used for experimental studies. Cells were incubated with hAAT (Prolastin®), doxycycline or both agents in combination for 6h and then stimulated with LPS (1µg/ml) or TNF-α (10ng/ml). 16-20 hours after stimulation, IL-6 secretion into the culture medium was determined by ELISA.

Interleukin-6 Assay

Murine IL-6 in culture medium of NIH/3T3 cells was determined by standard ELISA according to manufacture instructions (eBioscience, Inc., San Diego, Ca.). The level of quantification was 4 - 500 pg/ml.

Statistical Analysis

Analysis of the data was performed using GraphPad Prism 4.0 (GraphPad Software). Each data set was tested for variance and normality. Based on the results, the appropriate parametric or nonparametric tests were applied. $P < 0.05$ was considered statistically significant.

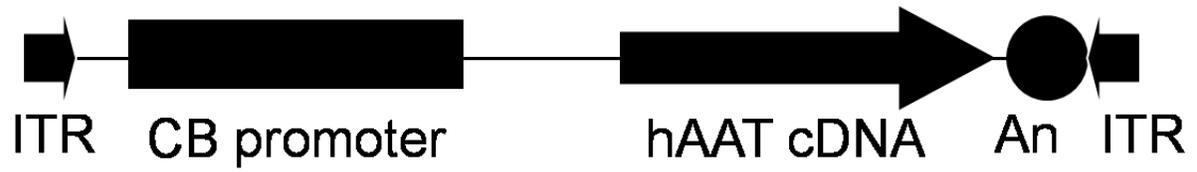


Figure 2-1. Vector construct of rAAV-CB-hAAT. The construct is flanked by two AAV inverted terminal repeats (ITR) and consists of a hAAT gene under the control of a CB-promotor followed by polyadenylation site (An).

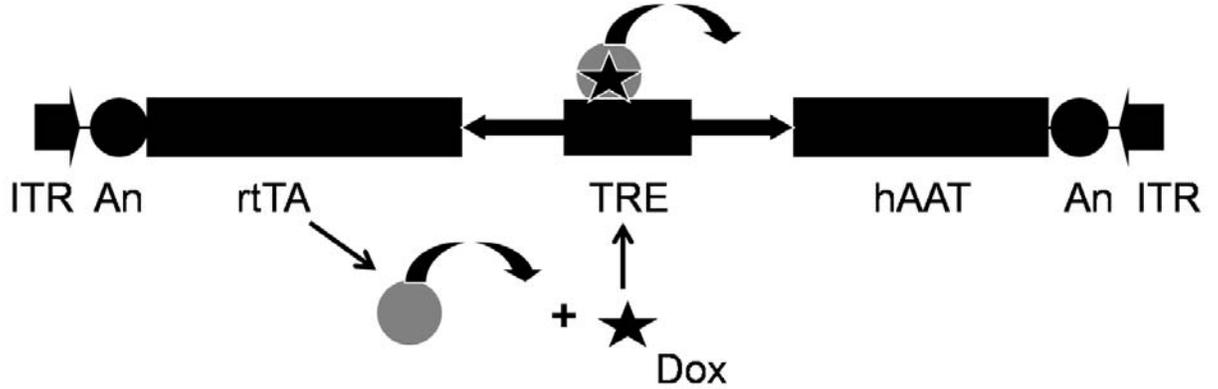


Figure 2-2. Vector construct of rAAV-tet-on-hAAT. Bidirectional promoter that contains tetracycline response elements (TRE) flanked by two mini-CMV promoters controls hAAT and rtTA genes. In the presence of doxycycline (Dox), rtTA will bind to the TRE and activate hAAT and rtTA genes. The rtTA gene serves as a positive regulatory system for hAAT gene transcription. An = polyadenylation site

CHAPTER 3
ALPHA-1 ANTITRYPSIN PROTEIN AND GENE THERAPIES IN COLLAGEN INDUCED
ARTHRITIS MODEL

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease affecting about 1% of the population in the United States. Recent studies have suggested that RA results from autoimmune self destruction and imbalance between inflammatory and anti-inflammatory network and extensive research over the last decades revealed new treatment strategies. These mainly include biologics targeting key players of synovial inflammation such as TNF- α and IL-1 β which have shown promising results in a number of patients. However, this new generation of cytokine targeting drugs does have serious side effects such as increased risk of infections, concerns about malignancies and other adverse outcomes. Therefore development of safe and effective treatment approaches is desirable.

Our previous studies in NOD mouse model showed that human alpha-1 antitrypsin (hAAT) gene therapy decreased levels of insulin auto antibodies, attenuated cellular autoimmunity and prevented autoimmune diabetes, implying that hAAT may be used for other autoimmune diseases (110). In the present study we intended to elucidate the feasibility of hAAT for the treatment of RA. We hypothesize that hAAT as a multi-functional protein with anti-inflammatory, immunoregulatory and tissue protective properties displays therapeutic potential for RA. To test our hypothesis we developed hAAT protein as well as adeno-associated virus mediated gene therapy in collagen induced arthritis, a commonly used, well established mouse model for RA.

The first study described in this chapter employed hAAT protein therapy as a convenient approach to determine the potential of hAAT in arthritis therapy. Human AAT protein was

delivered intraperitoneally into DBA/1 mice and arthritis development was evaluated according to paw swelling and joint deformity.

AAT protein therapy in humans is both expensive and inconvenient due to the necessity of repeating injections, therefore we also evaluated an alternative approach using rAAV serotype 8 to deliver the hAAT gene.

Since antibodies against type II collagen are essential for disease development in CIA mice, and it has been shown that inhibition of their development is accompanied with a reduction of disease severity, we also tested the effect of hAAT therapy on autoantibody development in CIA mouse model (114, 115).

Experimental Design

Protein Therapy Study

At the age of 8 weeks, arthritis was induced in DBA/1 mice by two bCII injections as described in Material & Methods. One group of mice (n=9) received hAAT protein injections twice/week starting six days before the first bCII immunization, another group (n=7), served as the control group and received saline injections (Fig.3-1).

Arthritis evaluation was started after the second collagen injection on day 21 and was performed by a blinded investigator three times per week. The evaluation was based on a well established grading scale for arthritis development as described in Materials & Methods. Serum samples were taken every other week to determine hAAT protein level as well as anti-hAAT and anti-CII antibody levels by ELISA.

On day 70 after immunization, mice were sacrificed and splenocytes were isolated to perform T-cell proliferation assay.

Gene Therapy Study

DBA/1 mice (n=10) were IP injected with rAAV8-CB-hAAT vector (2×10^{11} particles/mouse, Figure 2-1). The control group (n=10) received a single saline injection at the same time (Figure 3-7). Two weeks later, arthritis was induced by two bCII injections as described in Materials and Methods.

As for protein therapy, arthritis evaluation started after the second bCII immunization and was performed three times a week by a blinded investigator using the 0 to 4 scoring scale for arthritis development. Every other week, serum samples were collected to measure hAAT protein as well as anti-hAAT and anti-CII antibody levels by ELISA. Mice were sacrificed on day 56 after the first immunization.

Results

Arthritis Development in DBA/1 Mice is Suppressed by hAAT Protein Therapy

In order to investigate the effect of hAAT on development of arthritis, we first examined the feasibility of hAAT protein therapy in CIA mouse model. Administration of hAAT resulted in sustained high levels of hAAT in mouse serum (Figure 3-3A). Although anti-hAAT-antibodies were detected (Figure 3-3B) serum levels of hAAT did not decrease over time.

A few days after the second immunization with bCII (day 21), mice in control group developed arthritis in multiple joints, which was manifested by redness, severe joint swelling and joint stiffness as well as ankylosis as the disease progressed. The severity of arthritis as measured by the arthritic score rapidly increased in control group until a maximum score of 15 ± 1 was reached at day 63. Interestingly, the development of arthritis was suppressed in hAAT treated group as indicated by a significantly reduced clinical score (Figure 3-4A) and lower incidence of severe arthritis (score >3) ($p = 0.0025$, logrank test, Figure 3-4B). Moreover, mice in hAAT treated group had significantly delayed onset of arthritis compared with control group.

The clinical signs of severe arthritis started on day 47.3 ± 8.7 in hAAT treated group compared to day 36.0 ± 5.8 in control group ($p = 0.01$ by students t-test). In addition, the numbers of arthritic paws were also significantly reduced in hAAT treated group ($p < 0.05$ by Fisher's exact test, Figure 3-4C). These results showed that treatment with hAAT protein led to a delayed arthritis onset and amelioration of disease progression in CIA mouse model.

Inhibition of T-Cell Proliferation by hAAT Protein Therapy

Since CIA is a T-cell-mediated autoimmune disease, the effect of hAAT on T-cell function was examined by a T-cell proliferation assay. As shown in Figure 3-5, ConA – induced proliferation of splenocytes from hAAT protein treated group was significantly lower than that of control group ($p < 0.05$).

Human AAT Protein Therapy Reduces the Levels of Anti-bCII and Anti-mCII Autoantibodies

It has been shown that high levels of serum anti-collagen II autoantibodies are pathognomonic and associated with the development of arthritis (115, 116). To test the effect of hAAT on autoantibody production, we evaluated the levels of anti-CII autoantibodies in total Ig, and IgG1 and IgG2a subclass at early (day 35) and late (day 49) stage of the disease. As shown in Figure 3-6A, hAAT treatment did not result in a significant change of total autoantibody levels against bCII (total anti-bCII-Ig). However, hAAT treatment significantly reduced the pathognomonic IgG2a (anti-bCII-IgG2a) levels at day 35 (Figure 3-6B), and increased IgG1 (anti-bCII-IgG1) levels at day 49 (Figure 3-6C), consistent with a shift from a Th1 to Th2 response. Interestingly, total Ig autoantibodies against endogenous mouse collagen II (total anti-mCII-Ig) were significantly lower in hAAT protein treated group than that in control group ($p < 0.05$) (Figure 3-6D).

Human AAT Gene Therapy Suppresses Arthritis Development

To further confirm our observation that hAAT is effective in suppressing arthritis, we used recombinant adeno-associated virus vector (rAAV) to deliver the hAAT gene. A single IP injection of rAAV8-CB-hAAT vector resulted in sustained levels of hAAT in the circulation (Figure 3-8A). Interestingly, we did not observe the development of antibodies to hAAT after AAV8 mediated gene delivery (Figure 3-8B, compare vs. Figure 3-3B in mice with hAAT protein therapy). Similar to the results from hAAT protein therapy, rAAV-mediated hAAT gene therapy significantly reduced arthritis development (Figure 3-9A) and delayed the onset of the disease in CIA mouse model as indicated by a reduced severe arthritis incidence in treatment groups compared to control ($p=0.028$ by logrank test, Figure 3-9B). We also observed fewer arthritic paws in mice receiving rAAV8-CB-hAAT compared to control ($p< 0.05$, Figure 3-9C).

Levels of Anti-CII Autoantibodies are Reduced in rAAV8-cb-hAAT Injected Mice

As shown in Figure 3-10, rAAV8-mediated hAAT gene therapy resulted in a significant suppression of anti-CII autoantibody production. The levels of total Ig anti-bCII (Figure 3-10A) and IgG2a anti-bCII (Figure 3-10B) were significantly reduced in hAAT gene therapy group. Although IgG1 anti-bCII levels (Figure 3-10C) were also lower in hAAT gene therapy group, the ratio of IgG2a anti-bCII to IgG1 anti-bCII (Figure 3-10D) was significantly decreased in hAAT gene therapy group, suggesting a shift from Th1 to Th2 skewed response. Importantly, hAAT gene therapy also reduced levels of autoantibodies against mCII and the ratio of IgG2a anti-mCII to IgG1 anti-mCII (Figure 3-10E-H).

Discussion

RA is a complex systemic autoimmune disease with unknown etiology. Although recently developed biologicals that target TNF-alpha have provided dramatic improvement in controlling disease activity in many patients, continued searches for more efficient and safer treatments are

still needed. In the present study we showed that hAAT, administered as protein or through rAAV8 mediated gene therapy, reduced anti-CII autoantibodies and significantly delayed arthritis development in a mouse model.

To date, this is the first report on beneficial effects of hAAT in treatment of arthritis. Although the exact mechanisms underlying the therapeutic effect remain to be further investigated, several possible mechanisms may be involved. One is through the inhibition of proinflammatory cytokine production. It is well known that various proinflammatory cytokines, including TNF- α and IL-1- β , play major roles in the pathogenesis of RA (117, 118). Strategies targeting these cytokines have proven to be effective in treatment of RA (21). Previous work done by Janciauskiene and her colleagues clearly demonstrated that hAAT inhibited LPS-induced TNF- α , IL-6 and IL-1 β production by human monocytes (94, 95). In addition, hAAT completely suppressed macrophage inflammatory protein-2 (MIP-2)/monocyte chemotactic protein-1 (MCP-1) gene expression in lung (119). hAAT also enhanced anti-inflammatory cytokine IL-10 production from monocytes (94).

It is possible that the effects of hAAT on cytokine production contribute to shift the immune response from Th1 to Th2 pathway and inhibit autoantibody production. In previous studies we showed that hAAT reduced anti-insulin autoantibodies (IAA) and attenuated cell-mediated autoimmunity (110, 120). Consistent with these results, this study shows that hAAT reduces T-cell proliferation and the levels of anti-CII autoantibodies. In addition, hAAT reduces the IgG2a/ IgG1 ratios of anti-CII autoantibodies (mCII and bCII), consistent with a shift of immune response from Th1 to Th2 pathway.

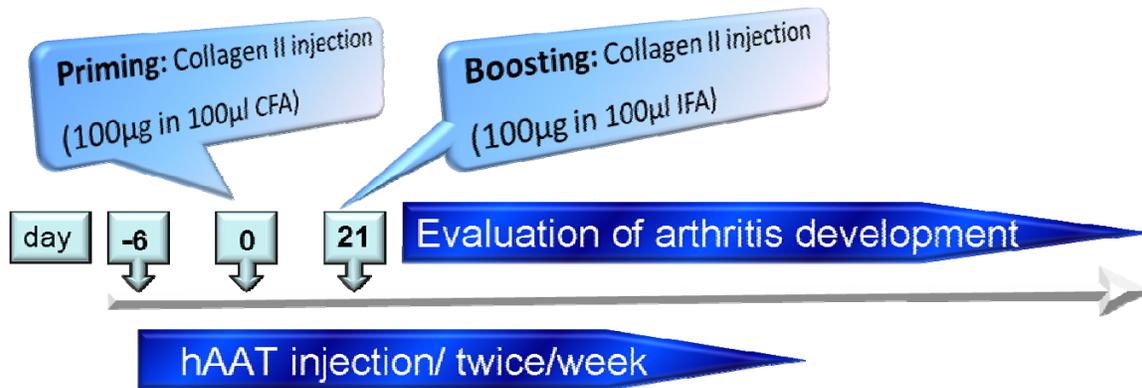
As a consequence of interfering with the cytokine/chemokine network, hAAT may also inhibit polymorphonuclear leukocyte (PMN) invasion into the joint. Churg et al. demonstrated

that hAAT inhibited silica-induced PMN influx into the lung and partially suppressed nuclear transcription factor B (NF- κ B) translocation and increased inhibitor of NF- κ B (I- κ B) levels in a mouse model of acute PMN mediated inflammation (119).

Another possible mechanism of hAAT suppressing arthritis development is through inhibition of proteinases to protect tissue injury and joint destruction. Human AAT is well known as a serine proteinase inhibitor (serpin). It inhibits trypsin, proteinase 3, neutrophil elastase, and cathepsin G, which are all involved in tissue injury. Human AAT can also reduce ischemia-induced apoptosis, inflammation, and acute phase response in the kidney (99). We have recently shown that hAAT directly inhibits caspase 3 activity and protects islet cells from cytokine and chemically-induced apoptosis (121, 122).

In the protein therapy studies, we used Prolastin[®], which is clinical grade of hAAT purified from human plasma. Repeated IP injection of hAAT induced strong humoral immune response against hAAT in DBA/1 mice (Figure 3-3B). It is possible that non-specific inflammation caused by repeated IP injection is responsible for inhibition of arthritis. In order to rule out this possibility, we performed rAAV8 mediated hAAT gene therapy. AAV serotype 8 vector is unique for this purpose because it can mediate long term and high levels of transgene expression in the liver and muscle, but is not able to transduce dendritic cells and has low immunogenicity (123, 124). Indeed, after a single injection of rAAV-CB-hAAT vector, sustained high levels of hAAT were detected in the circulation, while no detectable levels of anti-hAAT antibodies were present (Figure 3-8C) in contrast to mice that received hAAT protein therapy (Figure 3-3B). Importantly, we have observed similar protective effects and reduction of autoantibodies by both methods. These results strongly support our hypothesis that hAAT is able to reduce inflammation in autoimmune diseases, such as RA and type 1 diabetes.

In summary, our results from protein and gene therapy showed that hAAT is effective in suppressing arthritis in a mouse model of CIA. They indicate that hAAT has immunoregulatory and immunomodulatory effects and has great potential as a new treatment for RA. Future studies will focus on improvement of the therapeutic effect by optimizing the dose and timing of hAAT delivery, and by combination therapy with other anti-arthritic drugs.



Group	Dose	# of mice	Injection route
AAT (Prolastin®)	0.5mg/mouse/ twice week	9	i.p.
Saline	100µl	7	i.p.

Figure 3-1. Experimental design of hAAT protein therapy in CIA mouse model.



Severity 0



Severity 1



Severity 2



Severity 3



Severity 4

Figure 3-2. Severity grades of arthritis development in mouse paws. Arthritis development was evaluated based on a well established macroscopic scoring system. Solid arrows indicate swollen digits, the dotted arrow indicates early stages of necrosis.

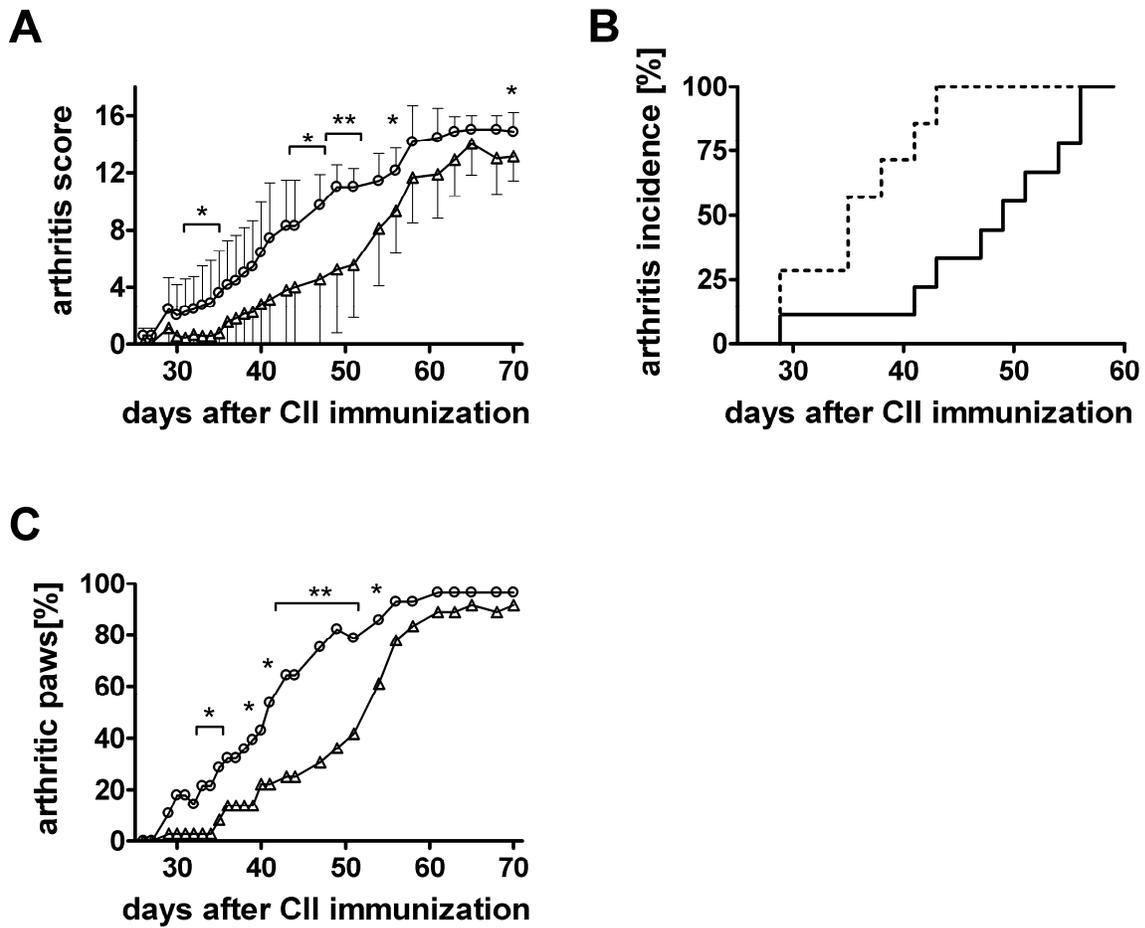


Figure 3-4. Anti arthritic effect of hAAT in CIA mouse model. A, Arthritis score. For each paw, 0 is normal and 4 is most severe arthritis. For each animal the maximum score is 16. Each line represents the scores from hAAT treated group (open triangles) or control group (open circles, mean \pm SD) * indicates $p < 0.05$, ** indicates $p < 0.01$, by Mann-Whitney U test. B, Incidence of severe arthritis. Mice were considered to be severe arthritic if arthritis score/mouse > 3 ($P = 0.0025$ by logrank test). Dotted line, saline injected control group; Solid line, hAAT treated group. C, Number of arthritic paws. Paws were considered to be arthritic when arthritis scores greater than 1. The total numbers of the arthritic paws in percent (arthritis score > 1) in hAAT treated group (open triangles) and control group (open circles) were plotted over time (mean \pm SD). * indicates $p < 0.05$, ** indicates $p < 0.01$ by Fisher's exact test.

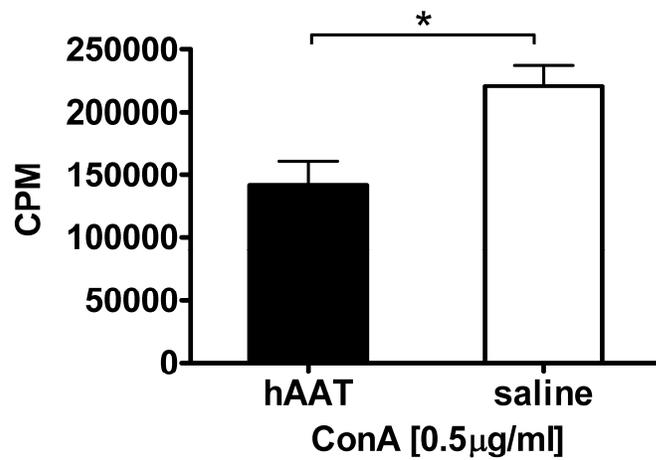


Figure 3-5. Protein therapy using hAAT reduces splenocyte proliferation. Splenocytes (4×10^5 cells/well, in 96-well plate) from hAAT treated group (black bar) and control group (open bar) were stimulated with ConA ($0.5 \mu\text{g/ml}$). Each bar ($n=3$) represents the average of ^3H -thymidine incorporation (count per minute, CPM) ($\text{mean} \pm \text{SD}$). $*p < 0.05$ by student t-test.

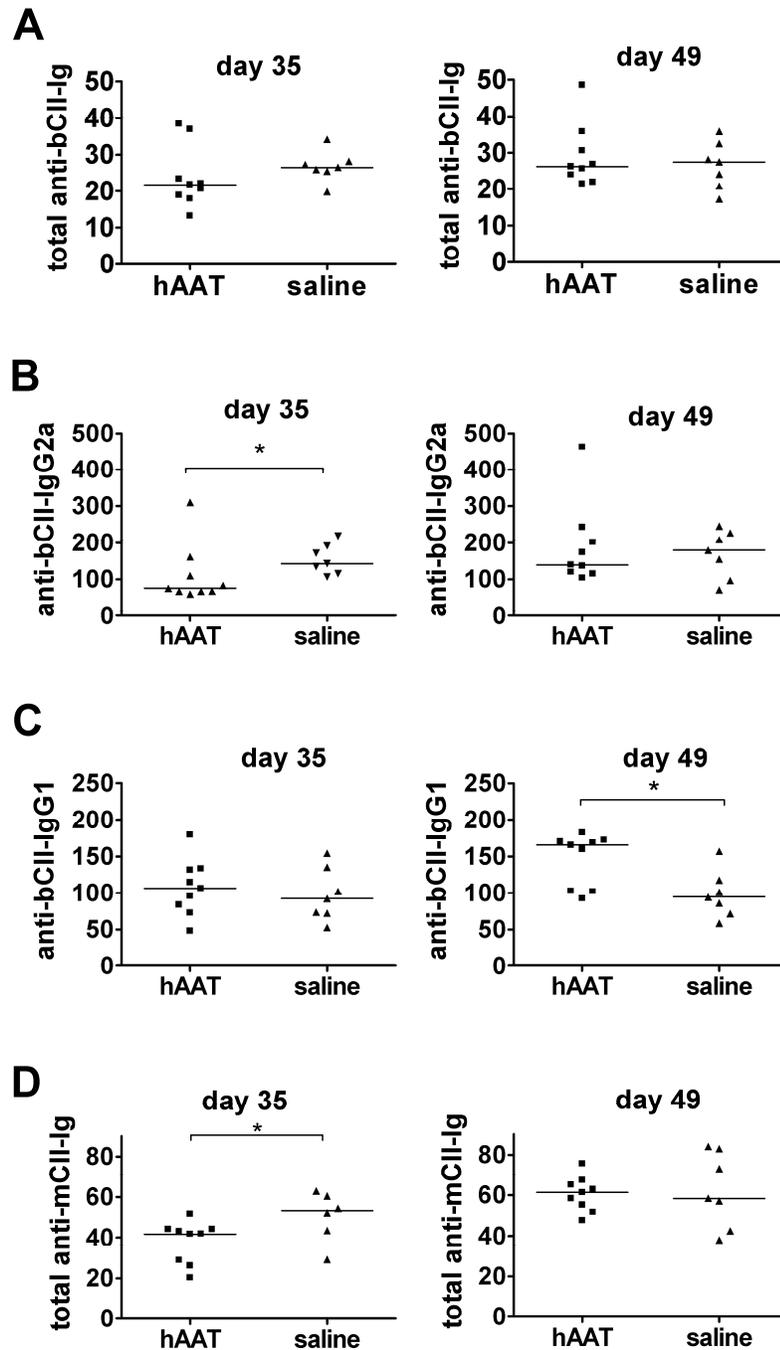
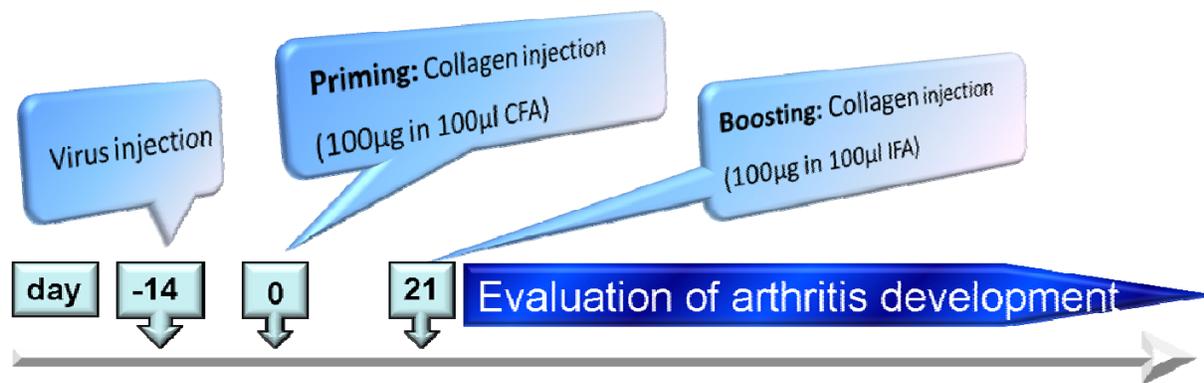


Figure 3-6. Anti-collagen II (CII) antibody levels after hAAT treatment. Anti-CII antibodies at day 35 and day 49 were tested by ELISA. Each dot represents the antibody levels (relative units) of an individual mouse. The horizontal line indicates median. A, Levels of total Ig antibodies against bCII (total anti-bCII-Ig). B, Levels of IgG2a against bCII (anti-bCII-IgG2a). C, Levels of IgG1 against bCII (anti-bCII-IgG1). D, Levels of total Ig antibodies against mCII (total anti-mCII-Ig). * $p < 0.05$ by Mann-Whitney U test.



Group	Titer	# of mice	Injection route
rAAV8 -CB-hAAT	2×10^{11} p	10	i.p.
Saline		10	i.p.

Figure 3-7. Experimental design for AAV8-CB mediated hAAT gene delivery in CIA mouse model.

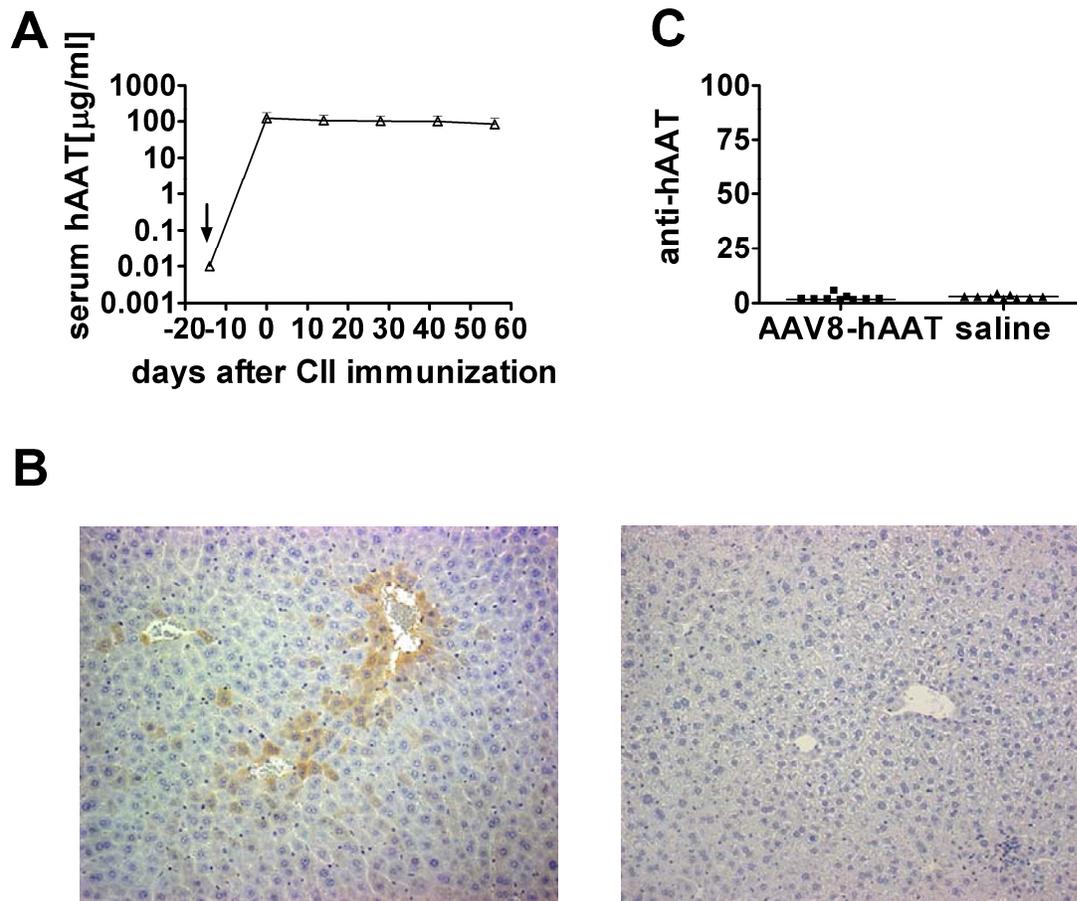


Figure 3-8. Stable and prolonged expression of hAAT after rAAV8-CB-hAAT vector injection. DBA/1 mice were intraperitoneally injected with rAAV8-CB-hAAT vector (2×10^{11} particles/mouse, $n=10$) or saline ($n=10$) at two weeks before immunization. Control group received saline. A, Serum hAAT protein in vector injected group was detected by ELISA (mean \pm SD). \downarrow indicates the injection time. B, Immunostaining for hAAT. *left*, representative liver section from hAAT gene therapy treated mice; *right*, representative liver section from saline injected mice. Brown stained cells express hAAT. Magnification: 200x. C, Anti-hAAT antibody levels. Serum anti-hAAT antibodies in vector injected group were undetectable by ELISA (at 1:50 dilution, day 56 after immunization, relative units). Each dot represents antibody level of an individual mouse.

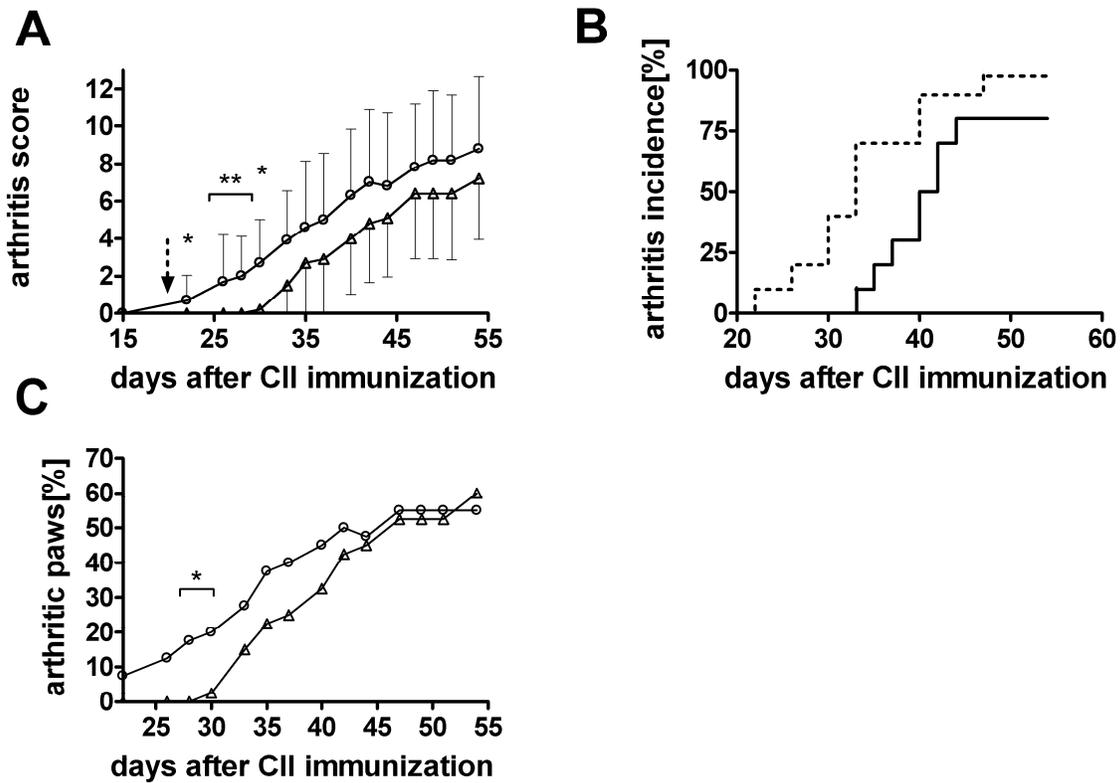


Figure 3-9. Human AAT gene therapy delays disease progression in CIA mouse model. A, Arthritis score. Each line represents the average scores from hAAT treated group (open triangles) or control group (open circles) (mean \pm SD). Arrow indicates the second CII immunization. * indicates $p < 0.05$, ** indicates $p < 0.01$ by Mann-Whitney U test. B, Incidence of severe arthritis. Mice were considered to be severe arthritic if arthritis score/mouse > 3 , ($p = 0.028$ by logrank test). C, Number of arthritic paws in percent. Paws were considered to be arthritic when arthritis scores greater than 1. The total numbers of the arthritic paws in hAAT treated group (open triangles) and control group (open circles) were plotted over time (mean \pm SD). * indicates $p < 0.05$ by Fisher's exact test.

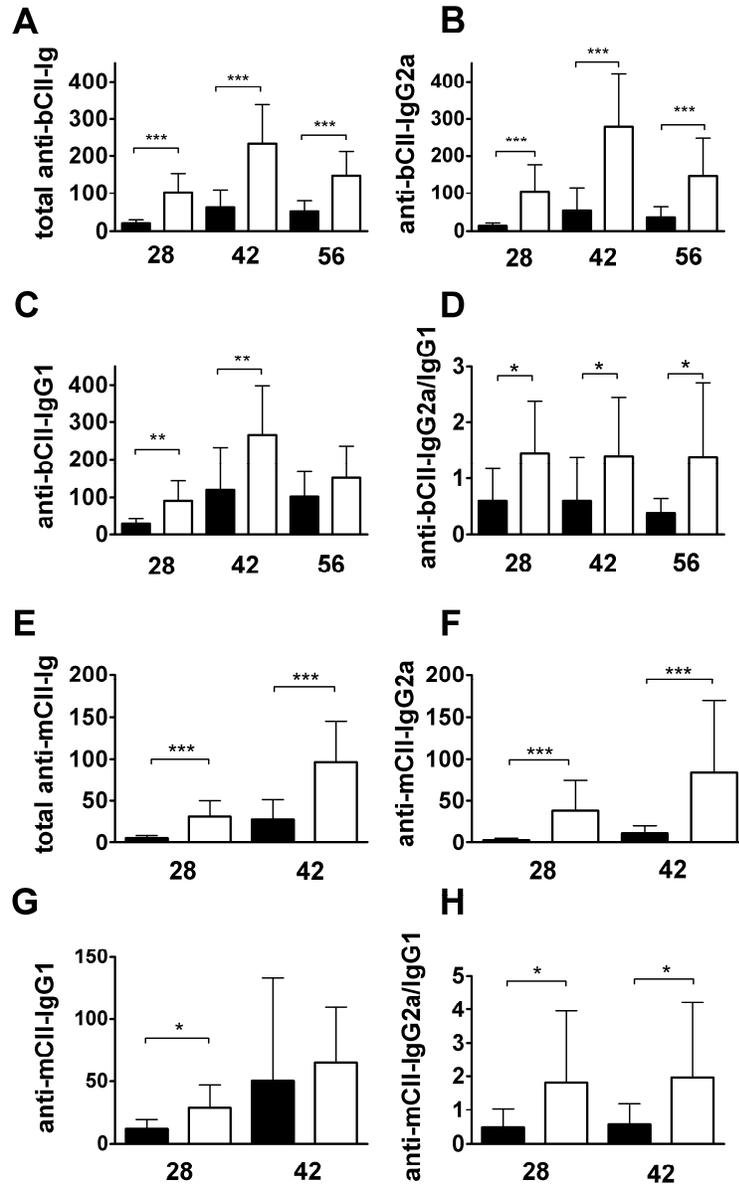


Figure 3-10. Effect of hAAT gene therapy on autoantibody production. Anti-CII antibodies at day 28, 42 and 56 were detected by ELISA. Black bars represent the average levels (n=10, mean±SD, relative units) of antibodies in hAAT gene therapy treated group. Open bars represent the average levels (n=10, mean±SD, relative units) of antibodies in saline injected group. A, Total antibodies against bovine CII (total anti-CII-Ig). B, Levels of IgG2A against bovine CII (anti-bCII-IgG2A). C, Levels of IgG1 against bovine CII (anti-bCII-IgG1). D, The ratio of anti-bCII-IgG2A to anti-bCII-IgG1 (anti-bCII-IgG2A/IgG1 ratio), E, Total antibodies against mouse CII (total anti-mCII-Ig). F, Levels of IgG2A against mouse CII (anti-mCII-IgG2A). G, Levels of IgG1 against mouse CII (anti-mCII-IgG1). H, The ratio of anti-mCII-IgG2A to anti-mCII-IgG1 (anti-mCII-IgG2A/IgG1). *indicates p<0.05, **indicates p<0.01, ***indicates p<0.001 by Mann-Whitney U test.

CHAPTER 4 COMBINATION THERAPY USING DOXYCYCLINE AND AAV8-TET-ON VECTOR EXPRESSING AAT

Introduction

As described in Chapter 1, rheumatoid arthritis is known as a complex disease with various factors being involved in disease development. Various cell types such as T-cells, B-cells, macrophages as well as fibroblasts participate in disease pathogenesis and they have the ability to secrete mediators which activate the complex network of a proinflammatory immune response (117). It has been shown that currently applied monotherapies of disease modifying drugs (DMARD'S) are ineffective in some patients and serious side effects can occur. Increased susceptibility to infections as well as malignancies are mainly caused by a suppressed immune system due to anti-TNF- α or anti IL-1 β therapy (125). In order to effectively treat rheumatoid arthritis with its complex pathogenesis, an advanced treatment strategy is necessary and it has been shown that combination therapy of antiarthritic drugs are highly effective (29).

The combination therapy of methothrexate and TNF- α or IL-1 β inhibitors has proven to be a promising alternative to established monotherapies because of improved treatment efficiency (21, 108, 126). It seems reasonable that targeting different mediators important for disease development increases treatment efficiency as well as may lower therapeutic doses and consequently side effects (29). Another study recently revealed that drugs, different from DMARD's, also have been shown to be highly effective in reducing arthritis development when combined with methotrexate. Combination therapy using doxycycline and methotrexate showed a significant American College of Rheumatology 50% improvement (ACR50) response in early seropositive rheumatoid arthritis patients when compared to methotrexate monotherapy (108). In our study, we propose to test the therapeutic effect of the combination of doxycycline and hAAT.

Because RA is characterized by recurrent periods of joint inflammation, it is desirable to limit therapeutic gene expression to the acute phases of inflammation. Constitutive over expression of transgenes can lead to detrimental effects in disease conditions. In order to control hAAT gene expression in our study, we delivered an AAV vector containing a doxycycline dependent tet-on gene expression system. This system enables us to control hAAT gene expression with doxycycline, which in addition has therapeutic properties.

Experimental Design

Cohorts of DBA/1 mice received either a single IP injection of rAAV8-tet-on-hAAT vector or saline. In addition mice in vector injected (n=9) or saline treated group (n=11) also received doxycycline ad libitum, administered with food (200mg/kg food). A control group (n=11) received a saline injection. Arthritis was induced 4 weeks after vector injection by two bovine collagen II injections as described in Materials & Methods (Fig.4-1).

Evaluation of arthritis was performed three times per week by a blinded investigator based on a well established arthritis scoring scale as described in Materials and Methods. Every other week, serum samples were collected to determine hAAT serum levels.

On day 56 after immunization mice were sacrificed. Liver was harvested for hAAT immunostaining and joints were collected and prepared for H&E staining to determine and evaluate histopathological changes.

To further evaluate the mechanism involved in hAAT and doxycycline mediated arthritis suppression, we conducted in vitro studies using mouse embryonic fibroblast cell line NIH/3T3. Simulating IL-6 secretion from activated synovial fibroblasts, a process involved in arthritis development, the cells were stimulated with LPS or TNF- α . In the presence of hAAT and/or doxycycline, IL-6 secretion into culture medium was evaluated by ELISA.

Results

Administration of AAV8-tet-on-hAAT and Doxycycline Resulted in Sustained Expression of hAAT in DBA/1 Mice

Our previous studies showed that AAV mediated hAAT gene delivery suppress arthritis in CIA model. To further improve the treatment effect we tested the feasibility of AAV mediated hAAT gene therapy in combination with doxycycline. We delivered an AAV vector, carrying the hAAT gene under control of the doxycycline inducible tet-on promoter (Figure 2-2). Four weeks after vector administration and starting doxycycline diet (200mg/kg food, ad libitum), a sustained level of hAAT protein was detected in mouse serum (Figure 4-2A). Consistent with our previous results, rAAV8 mediated gene delivery resulted in undetectable or weak immune response to the transgene product (hAAT). As seen in Figure 4-2B, only 25% of vector injected mice developed low titer anti-hAAT antibodies.

Gene Delivery Using rAAV8-tet-on-hAAT in Combination with Doxycycline Suppressed Arthritis Development in CIA Mice

As shown in Figure 4-3A, combination of doxycycline and hAAT results in a sustained suppression of arthritis development in mice ($p < 0.05$ by Mann-Whitney). When evaluating incidence of severe arthritis (clinical score/mice > 3), we observed, that mice receiving combination therapy developed significantly less severe arthritis compared to control group. Only combination therapy was able to significantly reduce incidence of severe arthritis development ($p < 0.05$ by logrank test, Figure 4-3B), whereas monotherapy using doxycycline alone did not result in a significant reduction.

Evaluation of arthritic paws revealed similar results. Only combination therapy was able to reduce the number of arthritis paws significantly compared to control and monotherapy ($p = 0.0011$ by Fisher's exact test adjusted for multiple comparison, Figure 4-3C). These results

showed that adding doxycycline to hAAT therapy significantly improved the treatment in reducing paw swelling and arthritis development.

Histopathological Changes in Mouse Joints are Improved by hAAT and Doxycycline Combination Therapy

To further determine the effect of the combination therapy, we evaluated joint sections from treatment and control groups. Figure 4-4 shows representative joint tissue sections from mice receiving combination therapy (Figure 4-4A-C) or saline injections (control group, Figure 4-4D-F). Combination therapy results in less inflammation and infiltration of immune cells into the joint space. Hyperplasia, pannus formation and cartilage erosion are significantly reduced in treatment group receiving doxycycline and hAAT compared to control group.

Joint tissue sections from all mice in respective groups were prepared and evaluated by two independent and blinded pathologists. The evaluation was based on grading the infiltration of immune cells into the joints, hyperplasia and pannus formation of synovial tissue as well as bone degradation. As presented in Figure 4-5, all histopathological criteria were significantly lowered in mice receiving combination therapy compared to mice receiving monotherapy or control ($p < 0.05$ by student's t-test). These results confirm the macroscopic observations described in Figure 4-4 and further underline that the combination therapy effectively suppresses immune cell infiltration, invasive, joint damaging tissue proliferation as well as cartilage and bone erosion.

Human AAT and Doxycycline Suppress IL- 6 Expression from NIH/3T3 Cells

To evaluate the mechanism of the anti-arthritic effect we observed after combination therapy we tested the effect of hAAT and doxycycline on disease associated IL-6 secretion from fibroblasts.

Following incubation with different concentrations of hAAT, ranging from 0.1mg/ml to 2.0 mg/ml, LPS induced IL-6 release was significantly reduced in a dose dependent manner

(Figure 4-6A). Interestingly, we did not observe this effect after incubation with hAAT and TNF- α induced IL-6 release (Figure 4-7A), indicating that the hAAT inhibitory effect may be more specific for the LPS signaling pathway.

After incubating cells with different doses of doxycycline, LPS or TNF- α induced IL-6 expression was significantly reduced in the presence of 100 μ g/ml doxycycline (Figure 4-6B and Figure 4-7B). Simulating the combination therapy applied in the presented study, we were also able to show that IL-6 concentration in culture medium was significantly reduced when cells were stimulated with LPS and incubated using combination of hAAT (1.0mg/ml) and doxycycline (10 μ g/ml and 100 μ g/ml) compared of hAAT alone (Figure 4-6C). The presence of hAAT (1mg/ml) and doxycycline (100 μ g/ml) also resulted in significant reduction of TNF- α induced IL-6 secretion into culture medium compared to hAAT alone (Figure 4-7C). This effect is probably due to the presence of doxycycline, since hAAT alone did not suppress TNF- α induced IL-6 expression (Figure 4-7D).

As summarized in Figure 4-6D, hAAT or doxycycline alone reduced LPS induced IL-6 secretion from fibroblast cells. This effect was further significantly enhanced by using a combination of these agents.

Discussion

Finding an effective treatment for rheumatoid arthritis is very challenging and until today it is not possible to cure the disease. One reason is certainly that it is still unknown what exactly triggers the disease onset. Since RA is driven by interaction of various immune cells, including T-cells, B-cells and macrophages, communicating through a network of cytokine and chemokines, it is challenging to determine what kind of intervention is effective in order to restore joint homeogenesis without causing unwanted side effects. Current treatments targeting cytokines such as TNF- α and IL-1 β are promising approaches and have shown to be highly

effective in clinical settings (29). However, since we are now able to examine long term effects of the new treatment strategies, it became obvious that they also have their drawbacks. Anti-TNF- α therapy failed to be effective in some patients accompanying with occurrence of side effects such as increased risk of infection, concerns about malignancies and other adverse outcomes (127-131).

Several studies have shown, that combination therapies, using drugs which target different pathways of disease pathogenesis are able to improve RA outcome significantly and therefore indicate to be a promising alternative treatment option (126). This strategy may also result in reduction of therapeutic dose accompanied with reduction of unwanted side effects. In our study we tested a combination therapy using hAAT and doxycycline. Both agents employ anti-inflammatory and immunomodulatory effects and we were able to show that they are effective when used as monotherapy. According to our hypothesis their treatment effect will be improved when used in combination. Doxycycline as a potent MMP inhibitor is able to reduce protease activity in the joint and also effectively suppresses proinflammatory cytokine release in vivo and in vitro (100, 101, 107). Similar, hAAT has been shown to reduce TNF- α , IL-1 β and IL-8 mRNA and protein expression in LPS stimulated macrophages (94, 132). Our study revealed that combination therapy using hAAT and doxycycline effectively suppresses arthritis development and is more effective than respective monotherapies.

Since RA is known as a chronic disease with recurrent active phases, characterized by acute inflammation, it is desirable to limit drug exposure to the active disease state. Employing gene therapy for this kind of disease characteristics has advantages because vector systems with controllable gene expression are available. Their introduction into RA therapy may also reduce side effects and minimize drug exposure during non acute disease states.

In accordance to the success of combination therapy in RA patients our study aimed to develop a unique combination therapy with a doxycycline dependent gene expression system. The uniqueness is, that doxycycline not only controls gene expression but also employs therapeutic potential. It not only targets the ongoing inflammatory autoimmune response during arthritis development but also turns on anti-inflammatory hAAT gene expression from the delivered AAV vector containing the tetracycline dependant gene expression system.

In order to further investigate the mechanism which leads to the suppression of CIA development in our study, we performed in vitro studies to test the ability of hAAT and doxycycline to reduce cytokine release from fibroblasts. Fibroblasts like synovial cells are key sources for proinflammatory cytokines, prostaglandins, metalloproteinases as well as other mediators of inflammation in the synovial cavity and are considered to be a major mediator of pathologic RA joint processes (133).

It has been shown that fibroblasts are a main source of IL-6 in RA patients and increased IL-6 activity correlates with elevated serum levels of acute phase reactants and other signs of inflammation such as fever and anemia (134). IL-6 has also been implicated as a major factor for the generation of auto antibodies (including rheumatoid factor). It also activates T-cells by inducing IL-2 production and IL-2 receptor expression and may act synergistically with IL-2 in driving the differentiation of T-cells into cytotoxic T-cells (135, 136). Playing an important role in establishing the RA synovial fibroblast (SF) phenotype, it increases SF proliferation in conjunction with its soluble receptor in the synovium of RA patients (137). Arthritis development was significantly suppressed in IL-6 deficient CIA mice indicating its major role in arthritis development(138). Targeting IL-6 is also effective in CIA mouse model after administration of a rat-anti-murine IL-6R monoclonal antibody early after arthritis induction as

shown by Takagi et al (139). Anti-IL-6 strategy is already successfully introduced in treatment of human RA. Tocilizumab, a humanized murine antibody to IL-6R which binds membrane associated as well as soluble IL-6R and therefore inhibits IL-6 from binding, shows promising potential for RA treatment in humans (29).

In the present study, we showed that both, hAAT and doxycycline are able to suppress LPS induced IL-6 release from fibroblasts in vitro, indicating a possible mechanism of our therapy for inhibition of arthritis development. Using a combination of both agents further suppressed the IL-6 production. These observations are in accordance with the results we received from the animal studies in which combination therapy using hAAT and doxycycline was more efficient in suppressing arthritis development compared to monotherapies.

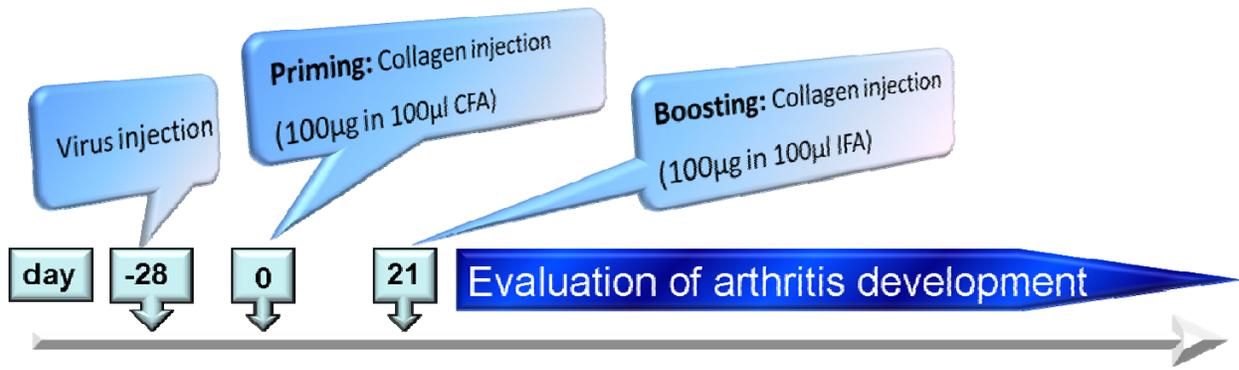
Our results also showed that hAAT alone was not able to suppress TNF- α induced IL-6 production in NIH/3T3 cells. This indicates that the suppressive effect of hAAT on LPS induced IL-6 production may be more specific for the LPS induced cell signaling pathway. Consistent with our observations, Nita et al. have shown that hAAT was able to decrease CD14 and Toll-like receptor 4 (TLR4) expressions in human monocytes in vitro (132). CD14 and TLR4 are important cell surface proteins of the LPS induced signaling pathway.

Interestingly, we also revealed, that in contrast to hAAT, doxycycline was able to suppress not only LPS induced IL-6 expression but also TNF- α induced IL-6 expression, indicating a different mechanism of action compared to hAAT. It has been shown by Cazalis and al. that pretreatment of macrophages with 10 μ M (= 5 μ g/ml) doxycycline prior to LPS stimulation resulted in a marked decrease in the phosphorylation of extracellular signal-regulated kinase (ERK1/2) (140). ERK is involved in both TNF- α and LPS mediated responses and therefore might be the target for doxycycline inhibition in our experiment (141, 142). Doxycycline also

downregulates proteinkinase C (PKC) pathways, as shown in a study of its effects on granuloma formation (143). TNF- α (141, 144-146) as well as LPS (147) pathways reportedly signal through PKC family members in different cell types, resulting in expression of proinflammatory cytokines.

We therefore suggest that the significant suppression of IL-6 release into the cell medium observed after treatment with the drug combination after LPS treatment might be due to inhibition of hAAT on CD14/TLR4 expression, as well as inhibition of ERK phosphorylation and downregulation of PKC pathways by doxycycline. Further studies have to be conducted to confirm this hypothesis and also its translation to in vivo settings.

In conclusion, our results show a new and promising approach to apply gene therapy for treatment of rheumatoid arthritis. Combining doxycycline and hAAT while being able to control gene expression of hAAT is a unique approach to target two different pathways involved in disease pathogenesis. The ability of hAAT and doxycycline to inhibit IL-6 expression from LPS stimulated fibroblasts also reveals a new property of this protease inhibitor. Future studies will focus on optimizing the treatment dose as well as using tissue specific AAV-vectors homing for disease affected tissues. In addition, delivering hAAT under self-limiting, inflammation-responsive promoter control is another promising approach for future arthritis treatment.



Group	Titer	# of mice	administration
rAAV8-cmv-tet-on hAAT	1x10 ¹¹ p + DOX (200mg/kg food)	9	i.p./orally
Doxycycline	DOX (200mg/kg food)	11	orally
Saline		11	i.p.

Figure 4-1. Experimental design for combination therapy of rAAV8 tet-on mediated hAAT gene therapy and doxycycline in CIA mouse model.

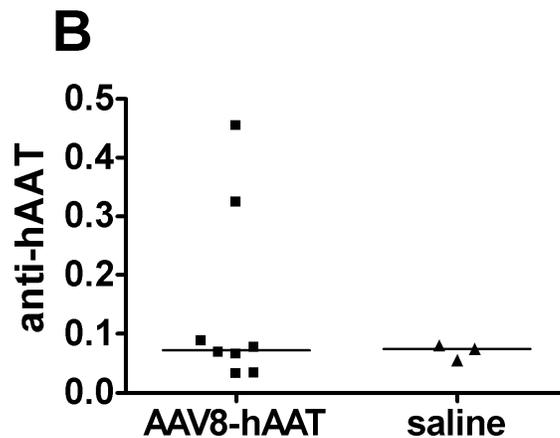
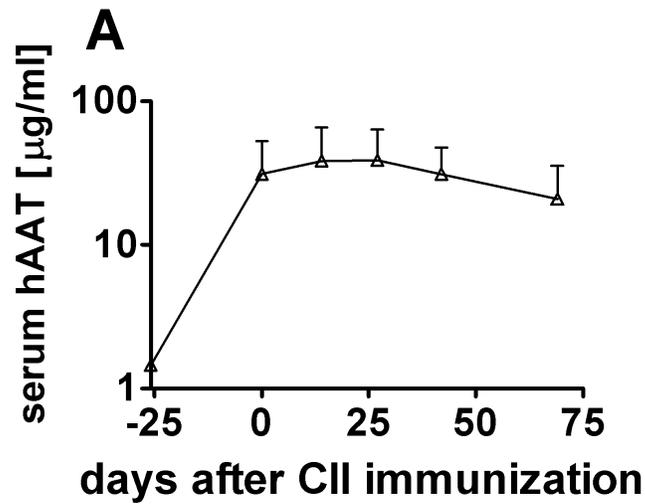


Figure 4-2. Human AAT serum and anti-hAAT level in DBA/1 mice received rAAV8-tet-on - hAAT and doxycycline. A, shown is hAAT level in mouse serum as determined by standard ELISA (mean \pm SD). rAAV8-tet-on-hAAT was injected intraperitoneally 28 days before CII immunization. B, anti-hAAT antibody level was determined by standard ELISA (at dilution 1:50). Each dot represents anti-hAAT level (measured as optical density (OD) at 490nm) of an individual mouse. Horizontal line represents median.

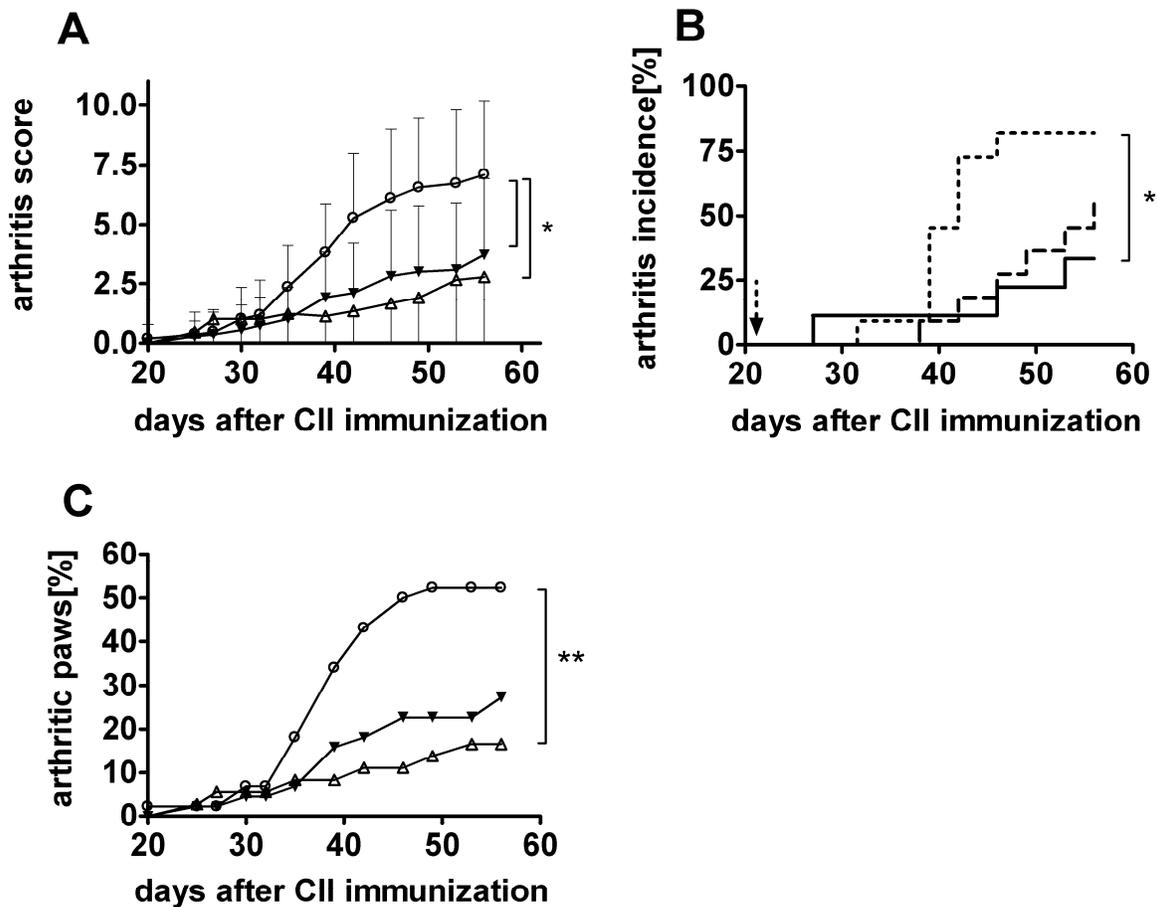


Figure 4-3. Anti-arthritis effect of combination therapy in CIA mouse model. A, Arthritis score. For each paw, 0 is normal and 4 is most severe arthritis. For each animal the maximum score is 16. Each line represents the scores from hAAT + doxycycline treated group (open triangles), doxycycline treated group (closed triangles) or control group (open circles) (mean \pm SD). * indicates $p < 0.05$ by Mann-Whitney U test. B, Incidence of severe arthritis. Mice were considered to be severe arthritic if arthritis score/mouse > 3 . Dotted line (short dots) represents saline injected control group; dotted line (long dots) represents doxycycline treated group; solid line represents hAAT + doxycycline treated group. * indicates $p < 0.05$ by log-rank test. C, Number of arthritic paws. Paws were considered to be arthritic when arthritis scores greater than 1. The total numbers of the arthritic paws in percent (arthritis score > 1) in hAAT + doxycycline treated group (open triangles), doxycycline treated group (closed triangles) and control group (open circles) were plotted over time. ** indicates $p < 0.01$, by Fisher's exact test adjusted for multiple comparison.

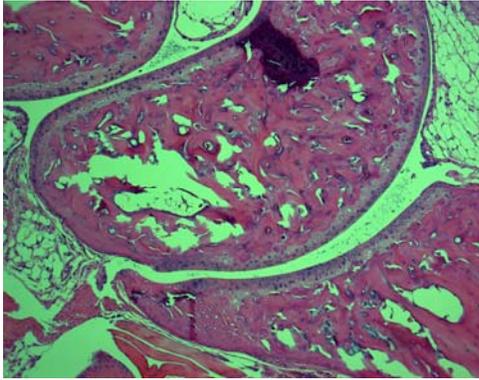
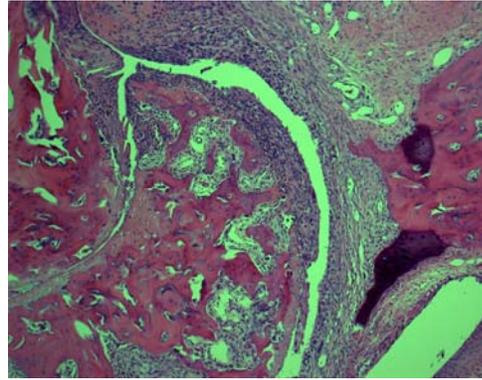
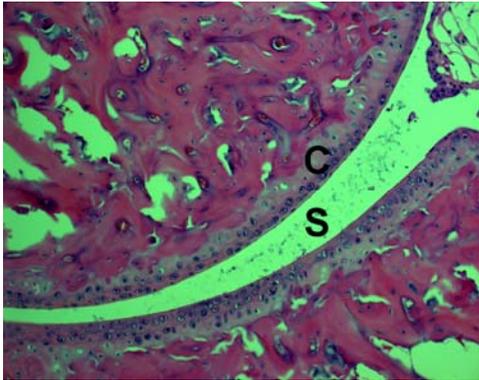
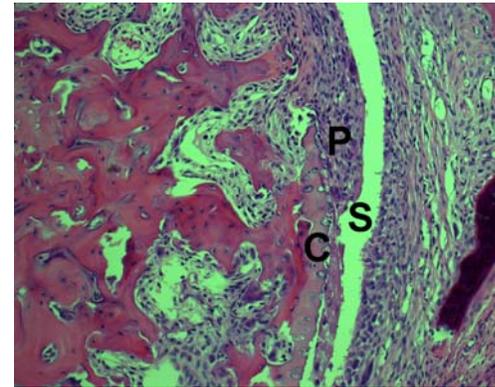
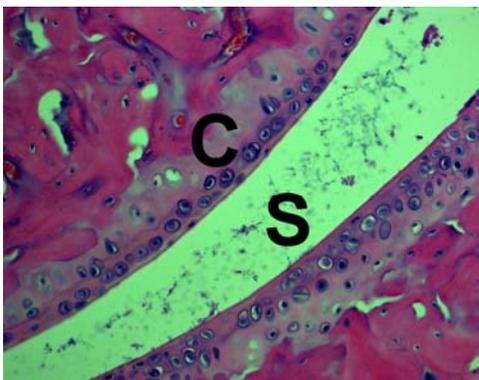
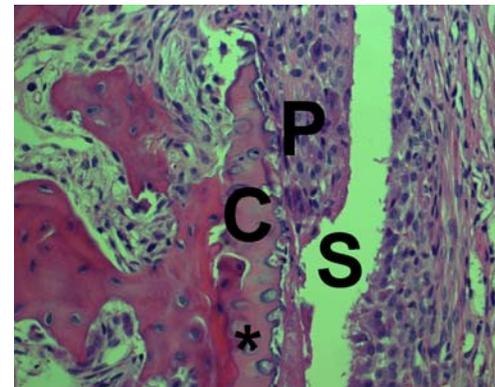
A**D****100x****B****E****200x****C****F****400x**

Figure 4-4. Tissue protective effect of combination therapy in CIA mouse model. Mice were sacrificed on day 56 after CII immunization, hind limbs were harvested and processed for histological assessment. A-C, representative joint section from mice receiving combination therapy. D-F, representative joint section from mice in control group (saline injection). Magnification: A, D: 100x; B, E: 200x; C, F: 400x. S= synovium, C= cartilage, P= pannus. * indicates cartilage erosion.

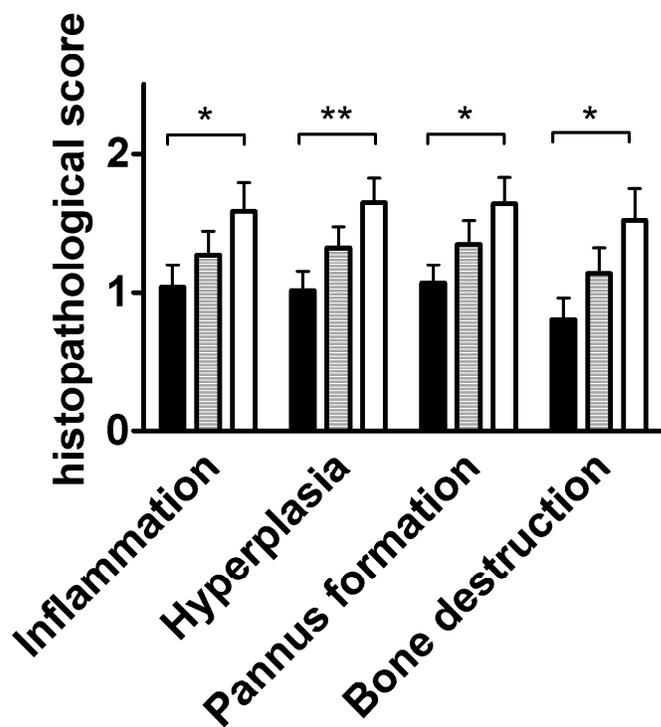


Figure 4-5. Histopathological evaluation of arthritis development. Mice in combination therapy group (black bars), doxycycline monotherapy group (striped bars) or control group (empty bars) were evaluated according to histopathological changes by two blinded pathologists. Each hind paw was evaluated based on a scale ranging from 0-4. (0: normal, 1: mild, 2: moderate, 3: severe, 4: very severe). The score was then averaged for each treatment group (mean±SD). *p=0.05, **p=0.01 by student's t-test.

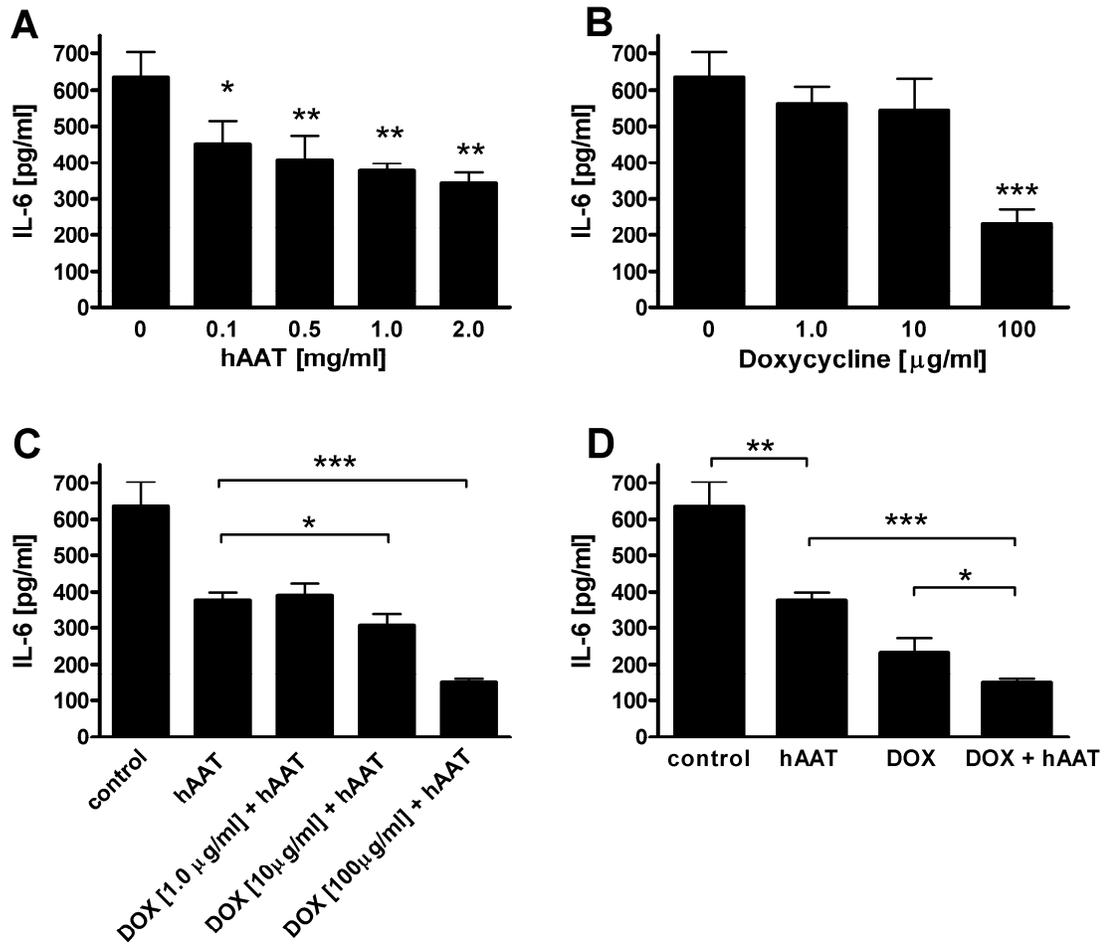


Figure 4-6. Effect of hAAT and doxycycline (DOX) on LPS-induced IL-6 release from mouse embryonic fibroblast cells (NIH/3T3). Cells were incubated with hAAT and/or doxycycline for 6h and stimulated with LPS (1 μ g/ml) for 20h. IL-6 release into the culture medium was determined by standard ELISA. A, IL-6 level in cell medium after incubation with indicated concentrations of hAAT. B, IL-6 level in cell medium after incubation with indicated concentrations of doxycycline. C, Incubation with hAAT (1mg/ml) and indicated concentrations of Doxycycline. D, Comparison of IL-6 level in cell medium after incubation with hAAT or doxycycline alone and in combination. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ to control group or as indicated using student's t-test.

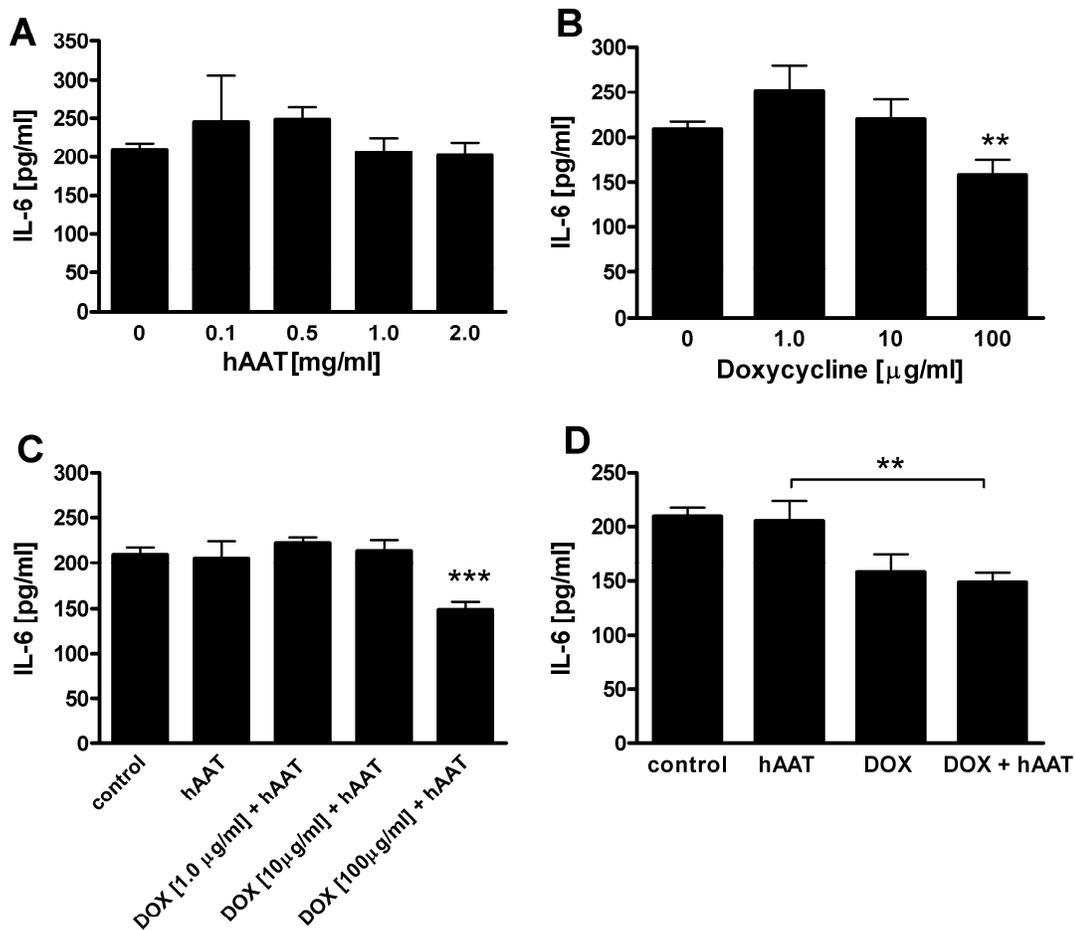


Figure 4-7. Effect of hAAT and doxycycline on TNF- α stimulated IL-6 release from mouse embryonic fibroblast cells (NIH/3T3). Cells were incubated with hAAT and/or doxycycline for 6h and stimulated with TNF- α (10ng/ml) for 20h. IL-6 release into cell medium was determined by standard ELISA. A, IL-6 level in cell medium after incubation with indicated concentrations of hAAT. B, IL-6 level in cell medium after incubation with indicated concentrations of doxycycline. C, Incubation with hAAT (1mg/ml) and different concentrations of doxycycline. D, Comparison of IL-6 level in cell medium after incubation with hAAT or doxycycline alone and in combination. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ to control group or as indicated using student's t-test.

CHAPTER 5
ADENO-ASSOCIATED VIRUS MEDIATED HUMAN AAT GENE THERAPY IN
PRISTANE INDUCED ARTHRITIS MODEL

Introduction

As we have reviewed in the previous chapters, rheumatoid arthritis is a very complex disease with multiple dysfunctional components of immunity in the pathogenesis. It has been reported that different cytokines such as IL-1, TNF- α , IL-6 and RANKL (receptor activator of nuclear factor κ B ligand) play important roles in disease pathogenesis indicating the complexity of the cytokine network involved (117). Observed heterogeneity of disease manifestations, clinical course and different treatment responses in RA patients further indicate the diversity of the disease and suggest that possible treatment approaches have to be examined very carefully. Due to this complexity and diversity it has been suggested that therapeutic strategies should be evaluated in different animal models.

Several animal models with different properties have been used for RA studies. Collagen induced arthritis and adjuvant induced arthritis show a high incidence as well as reliable onset and a robust and easy measurable poly-arthritic paw inflammation. MRL/lpr mice spontaneously develop mild arthritis with variable onset and incidence. For our studies we decided to use pristane induced arthritis because arthritis can be induced by a single i.p. injection of pristane and a prolonged and delayed clinical timecourse of joint inflammation can be observed. An extended and permanent period of active disease state enables us to better investigate the treatment effect.

In addition to the development of arthritis, pristane injected DBA/1 mice also develop lupus like autoimmunity characterized by autoantibody production against nuclear proteins.

Using this model for our studies elucidates the potential of hAAT and its combination therapy with doxycycline to suppress autoantibody production and will further reveal the therapeutic applicability of hAAT therapy in autoimmune diseases.

Experimental Design

Female DBA/1 mice received either a single i.p. injection of recombinant AAV8-cb-hAAT vector (n=20) or saline (n=20). The mice were randomly assigned to four groups (n=10 each). In addition to the AAV8-CB-hAAT vector, one group resembling the combination therapy also received a doxycycline containing diet. Other groups received rAAV8-CB-hAAT alone (hAAT monotherapy), doxycycline alone (doxycycline monotherapy) or a single saline injection (control group) (Fig.5-1). Two weeks after vector injection, mice were i.p. injected with a single dose of 0.5 ml pristane oil to induce arthritis and lupus like autoimmunity.

Every four weeks, serum samples were collected to determine hAAT levels and anti-hAAT as well as lupus related auto-antibodies. 12 weeks after pristane injection evaluation of arthritis development was started using the commonly accepted arthritis grading scale as described in Materials & Methods. At 34 weeks after pristane injection mice were sacrificed and proteinuria was determined in urine to test for Lupus Nephritis development.

Results

Serum Levels of hAAT and anti-hAAT in PIA Mouse Model Following rAAV8-cb-hAAT Mediated Gene Therapy

Based on our preliminary data, we decided to use rAAV8 mediated hAAT gene therapy to test the feasibility of hAAT therapy in PIA model. As shown in Figure 5-2A, 6 weeks after vector injection (or 4 weeks after pristane injection), serum level of hAAT protein serum level was about 40 μ g/ml in both groups which received hAAT gene therapy. Doxycycline, as expected did not have an effect on hAAT gene expression. We also evaluated the immune response to

hAAT by measuring anti-hAAT antibodies. As illustrated in Figure 5-2B, only few mice developed a minor immune response against the transgene.

Pristane Injection Results in Mild Arthritis Development in Treatment Groups

We evaluated the effect of hAAT monotherapy and doxycycline monotherapy as well as their combination therapy in the PIA mouse model. 12 weeks after pristane injection, mice started to develop arthritis characterized by joint redness and swelling. Arthritis development was then evaluated once a week until the experiment was terminated on week 34. As shown in Figure 5-3A, all groups showed slow and sustained arthritis development and reached the maximum average score between 4.5 and 7.5 at about 26 weeks after immunization. With a maximum possible score of 16, this indicates only mild arthritis development in all groups. Interestingly, we were not able to observe any differences based on clinical score between treatment groups. Similarly, when evaluating arthritis incidence in mice with score >1 as shown in Figure 5-3B we also were not able to observe any significant difference between groups. These results indicate that further optimization of the combination is required to get the therapeutic effect for arthritis in the model.

Lupus Nephritis Development Tends to be Suppressed by hAAT and Doxycycline Monotherapy

Besides arthritis development, pristane injection results in occurrence of lupus nephritis in mice which is characterized by proteinuria. We examined the effect of hAAT gene therapy on the induction of lupus nephritis. As shown in Figure 5-4, >1 proteinuria was detected in 7 of 10 mice from control group. Although not statistically significant, only 3 of 10 mice in hAAT monotherapy ($p=0.179$ by Fisher's exact test compared to control) and 2 of 10 in doxycycline monotherapy ($p=0.06$ by Fisher's exact test compared to control) indicated at least a trend of a suppressive effect on proteinuria of hAAT as well as doxycycline monotherapy. However,

combination therapy using hAAT and doxycycline did not show such convincing results. 4 of 9 mice developed >1 proteinuria in this group ($p=0.36$ by Fisher's exact test compared to control).

Effect of Mono- and Combination Therapy on Development of Autoantibodies

The development of lupus like autoimmunity in PIA model is characterized by production of autoantibodies against nuclear proteins such as anti-RNP, anti-rP, anti-Su, anti-chromatin, anti-ssDNA and anti-dsDNA. To examine the effect of hAAT gene therapy and its combination with doxycycline on autoantibody development we determined antibody levels in mouse serum 3 month and 8.5 month after pristane injection by ELISA. As shown in Figure 5-5 A,C, 12 weeks after pristane injection, only low antibody levels against chromatin and DNA were detectable. Figure 5-5B, D, E, illustrate, that although respective autoantibody levels were increased at 34 weeks compared to levels at 12 weeks, we did not determine significant differences between treatment groups.

Interestingly, Figure 5-6A shows, that combination therapy resulted in suppression of autoantibodies against Su protein at 12 weeks ($p= 0.0185$ by Mann-Whitney). In addition we were also able to observe a trend of anti-Su suppression at 34 weeks (Figure 5-6B, $p=0.144$ by Fisher's Exact test) indicating a suppressive effect of hAAT and doxycycline combination therapy on development of certain autoantibodies in PIA model.

Similar to anti-Su, autoantibody levels of anti-rP and anti-RNP increased over time (Figure 5-6 C-F). However, we were not able to determine any significant differences in anti-RNP and anti-rP autoantibody development between treatment groups and control.

Human AAT or Doxycycline Treatment Does Not Change Type of Antibody Response

To further characterize the antibody response that was elicited in our model and the ability of hAAT gene therapy or doxycycline treatment to modify this response, we determined the serum levels of different antibody classes. Depending on the kind of immune cells, involved in

the immune response, we will observe a characteristic pattern of antibodies in serum. Usually, pristane injection results in high titer IgG2a antibodies in mice resulting from a Th1 skewed response.

We determined levels of IgG1, IgG2a, IgG2b, IgG3 and IgM by ELISA. As seen in Figure 5-7, hAAT or doxycycline treatment did not change serum levels of these antibody classes.

Discussion

Animal models for rheumatoid arthritis are widely used to test the potential of anti-arthritis drugs for suppression of disease development because they resemble pathogenic and inflammation patterns similar to those seen in humans. Although animal models help us to understand the complex immunological and pathological patterns important for the disease, none of them exactly represents human RA. This suggests using different animal models to test for therapeutic efficiency. In addition, differences and diversity in severity, clinical symptoms of RA as well as response to treatment in patients also recommend examination of new anti-arthritis drugs in different animal models in order to cover a broad range of pathological manifestations.

The animal models we used in our studies, collagen induced arthritis and pristane induced arthritis, simulate human disease in different ways. The auto-antigen collagen II which induces CIA, elicits an autoimmune response which is characterized by a relatively short period of acute joint inflammation (max 6-7 weeks) followed by joint erosion and deformation. This model is commonly used when therapeutic effects on disease onset and early stages of disease progression are investigated. In contrast, pristane induced arthritis is characterized by a delayed, slow and chronic inflammation of the joint. The inflammation lasts 3-6 month and results in cartilage erosion and joint deformation after 5-6 month, similar to human RA which also progresses slowly. Using these two animal models therefore enable us to determine the effect of AAT on arthritis development from different perspectives. One being the acute inflammation in the

beginning of disease progression, the other is focusing on the long term effects while the disease progresses.

Our data using CIA model showed that both, protein as well as gene therapy using hAAT are effective. For PIA model we preferred the gene therapy approach since a single vector injection as it is performed for gene therapy is much more convenient than regular injections of protein. Especially when applied in PIA model in which disease progresses slowly over several month. We decided to use AAV serotype 8 to deliver the hAAT gene due to its constant gene expression and its promising effects on suppressing arthritis and autoantibody development as observed in the previous studies. Interestingly, the average hAAT level in PIA model was lower compared to the serum level in CIA model when injecting the same vector. This might be due to an increased antiviral type 1 interferon expression, commonly seen in response to pristane injection, and resulting in decrease of effective vector concentration.

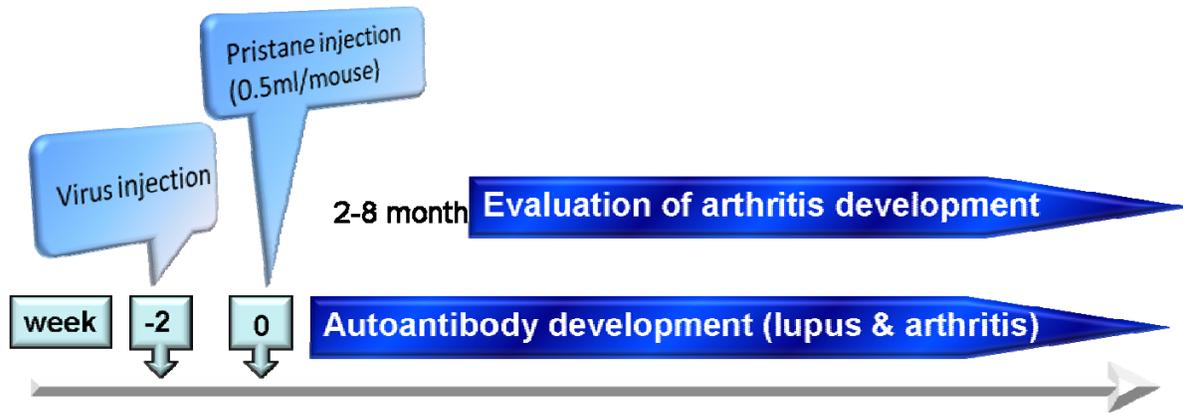
The immune response against hAAT seems to be slightly stronger than it has been seen in previous studies using AAV8 as a gene delivery vector. Three out of 20 mice developed elevated levels of hAAT antibodies (Figure 5-2). This might be due to an unspecific inflammatory response caused by the administration of pristane. In a sustained inflammatory state as a result of pristane injection, dendritic cells may more likely uptake the hAAT protein, which is then processed and presented to T-cells, eliciting the humoral response against hAAT.

Although we were able to detect elevated serum levels of hAAT, we did not observe an anti-arthritic effect in any of the applied therapies in PIA model. Neither hAAT nor doxycycline monotherapy nor the combination therapy was effective in this model. A possible reason could be that the hAAT level was not high enough for a sustained suppression of arthritis development over time. As seen in Chapter 3, hAAT effectively suppresses disease onset and development in

the early phase of arthritis but showed less therapeutic effect in inhibiting disease progression. Since PIA progresses slowly with major presentation of the chronic, disease progressing state, the hAAT effect on disease onset in this model might not be powerful enough to suppress arthritis progression in the long run. Another reason for the absence of a therapeutic effect could be that our therapies were effective to a certain grade, but since the severity of arthritis development in the control group was relatively low, the effects observed were undistinguishable. Our mice were held in SPF rooms and PIA progression may be inhibited due to lack of various factors such as ligands for pattern- recognition receptors, which are commonly abundant in normal environment and necessary for PIA development.

In addition to arthritis development, pristane injection also leads to development of lupus like autoimmunity characterized by elevated serum levels of autoantibodies against nuclear proteins in susceptible mice. As we learned from the previous studies, hAAT is able to inhibit autoantibody production in CIA mice. We therefore determined if hAAT also inhibits autoantibody production against nuclear proteins in PIA mouse model. Interestingly, the combination therapy showed the most promising results and revealed a reduction of autoantibodies against Su-protein. Unfortunately, we were not able to determine such an effect for any of the other lupus related autoantibodies anti-RNP, anti-ssDNA, anti-dsDNA, anti-Chromatin and anti-rP. The long term inhibitory effect of hAAT on autoantibody production might be limited and a suppressive effect can just be observed shortly after disease onset as seen in the previous studies described in chapter 3. This hypothesis is underlined by the ability of combination therapy to significantly suppress anti-Su development after 12 weeks and a less significant reduction after 34 weeks (Figure 5-6).

Further studies have to be conducted to eliminate uncertainties in the effect of hAAT in the PIA model. We need to further optimize disease onset and increase incidence and severity of arthritis development in this animal model. We are optimistic to observe a better hAAT treatment effect if we are able to increase overall disease severity in the control group. This will most likely also lead to more severe lupus development accompanied with higher levels of autoantibodies and increased incidence of proteinuria. In this case a hAAT effect may also more likely be detected.



Group	Titer	# of mice	administration
rAAV8-cb- hAAT + DOX	2x10 ¹¹ p + DOX (200mg/kg food)	10	i.p./ orally
rAAV8-cb- hAAT	2x10 ¹¹ p	10	i.p.
Doxycycline	DOX (200mg/kg food)	10	orally
Saline		10	i.p.

Figure 5-1. Experimental design of pristane induced autoimmunity in DBA/1 mice. Mice developed arthritis as well as lupus like autoimmunity several weeks after receiving a single i.p. injection of 0.5ml pristane.

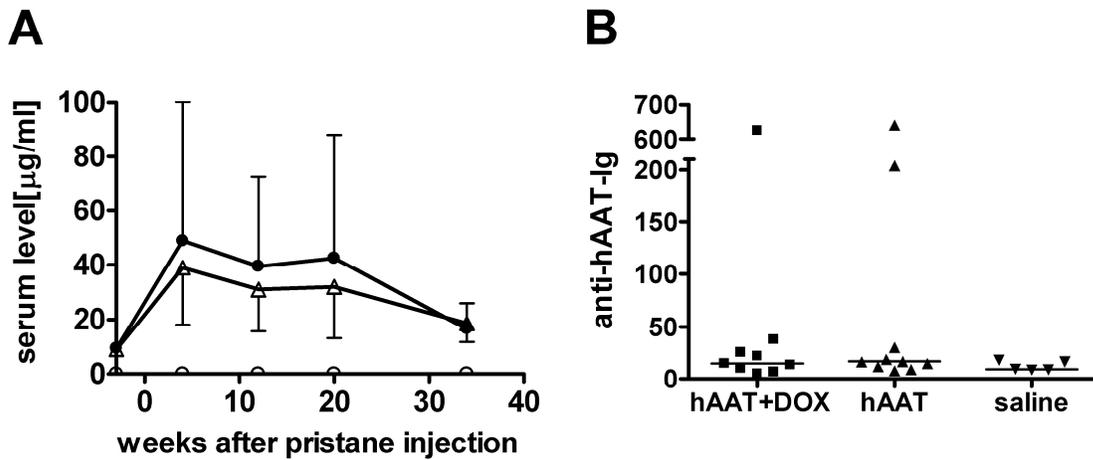


Figure 5-2. Human AAT and anti-hAAT antibody levels after rAAV8 mediated hAAT gene delivery in pristane induced mouse model. A, hAAT serum level after rAAV8-cb-hAAT gene delivery was determined by standard ELISA. Shown are levels of hAAT gene therapy alone (open triangles) and in combination with doxycycline (closed circles) (mean±SD). B, Serum antibody levels against hAAT were determined on week 34 after pristane injection. Shown are serum anti-hAAT levels of individual mice. Horizontal line represents median.

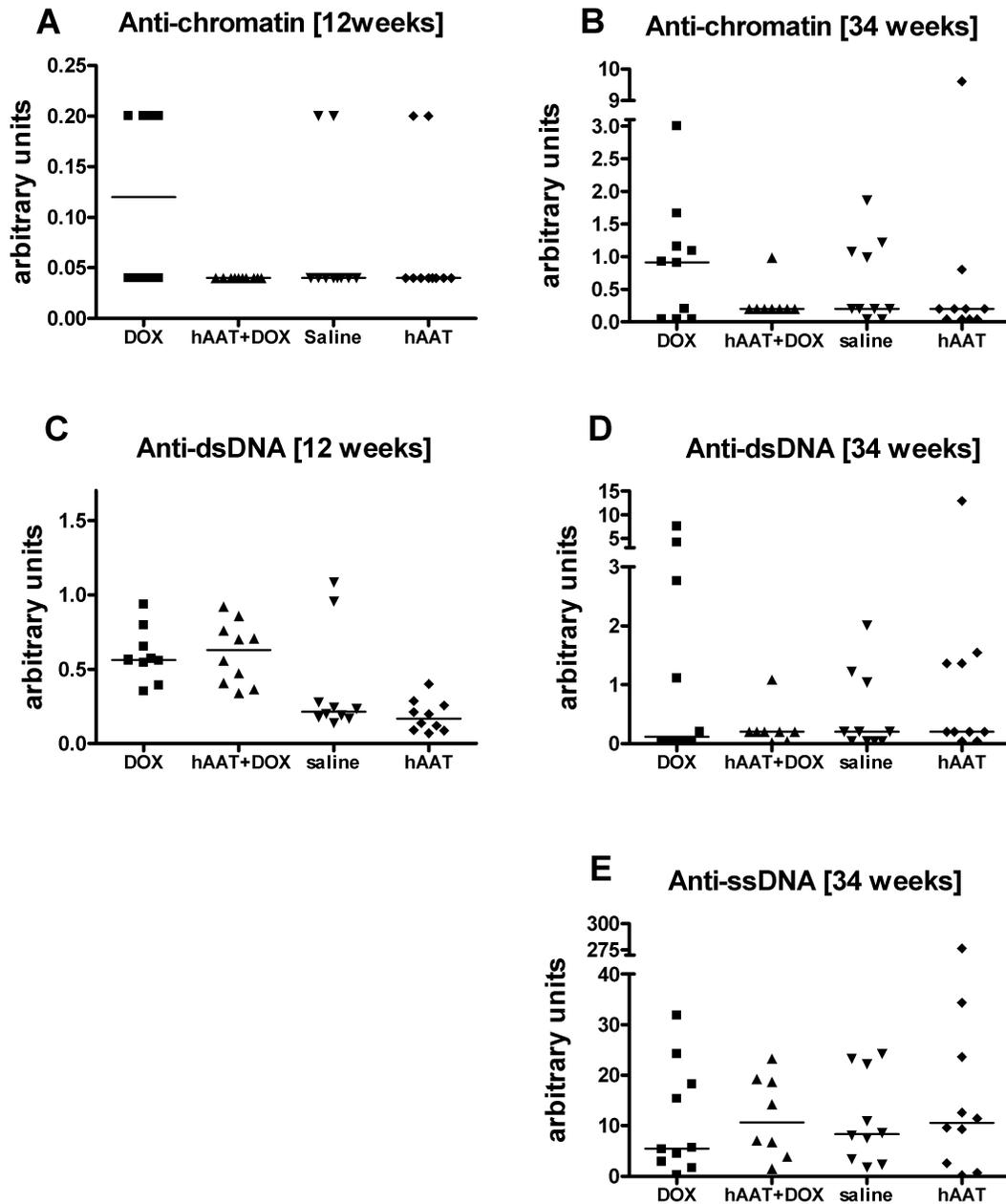


Figure 5-5. Autoantibody production in pristane treated DBA/1 mice after receiving different treatments. Serum levels of lupus autoantibodies were determined by standard ELISA. A,B, Serum levels of anti-chromatin antibodies were determined using ELISA for detection of reactivity with chicken erythrocyte chromatin at indicated timepoints. C,D, Sera from treatment or control groups were tested for presence of anti-double-stranded antibodies (anti-ds-DNA) at indicated time points. E, Antibodies against single stranded DNA (anti-ssDNA) were detected 34 weeks after pristane injection. Horizontal lines indicate median for each group.

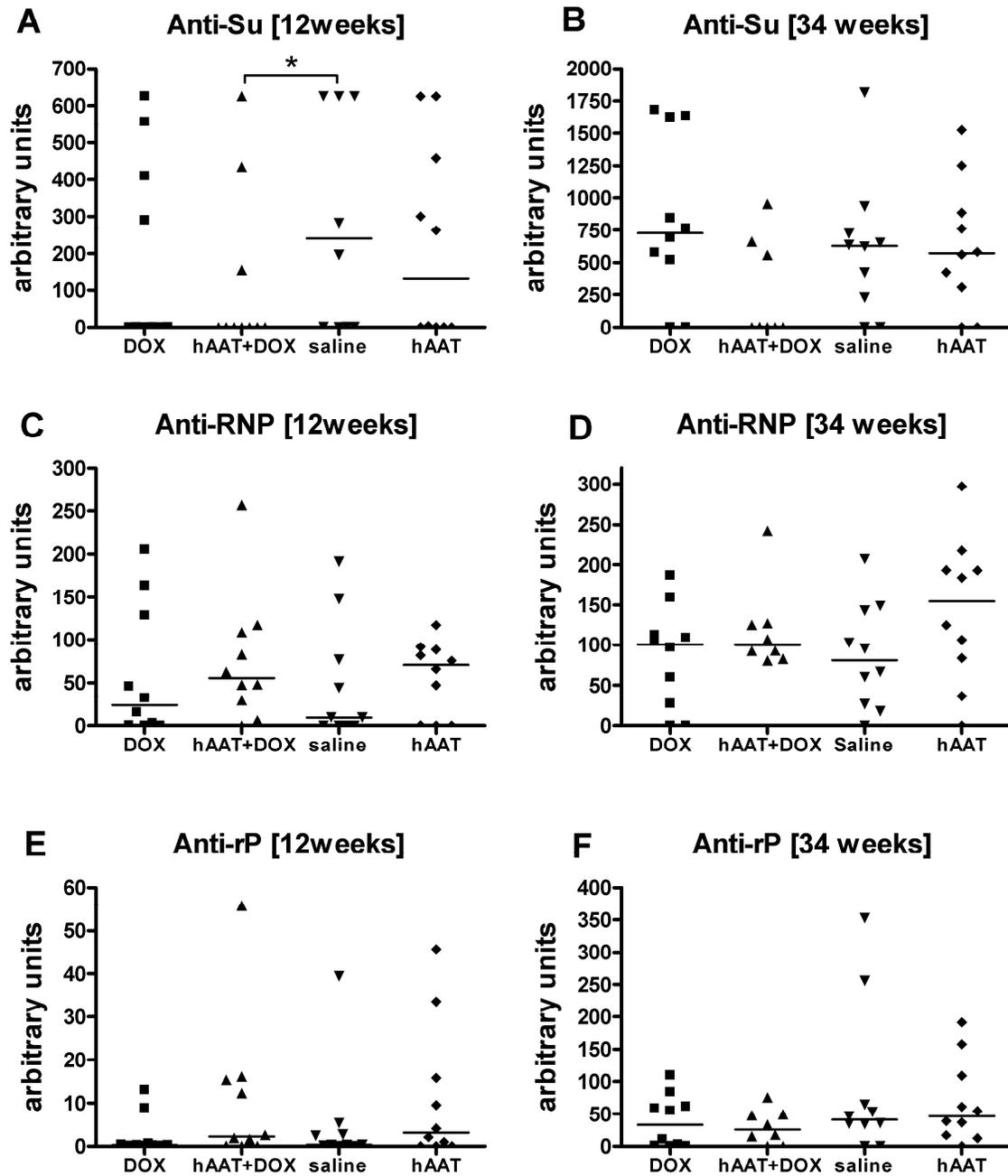


Figure 5-6. Production of lupus autoantibodies after pristane injection in DBA/1 mice. A,B, anti-Su antibodies; C,D, anti-nuclear RNP antibodies, E,F, anti-ribosomal P antibodies were measured by ELISA at indicated time points in saline control groups and the indicated treatment groups. Horizontal line represents the median in each group. * $p < 0.05$ determined by Mann-Whitney U test.

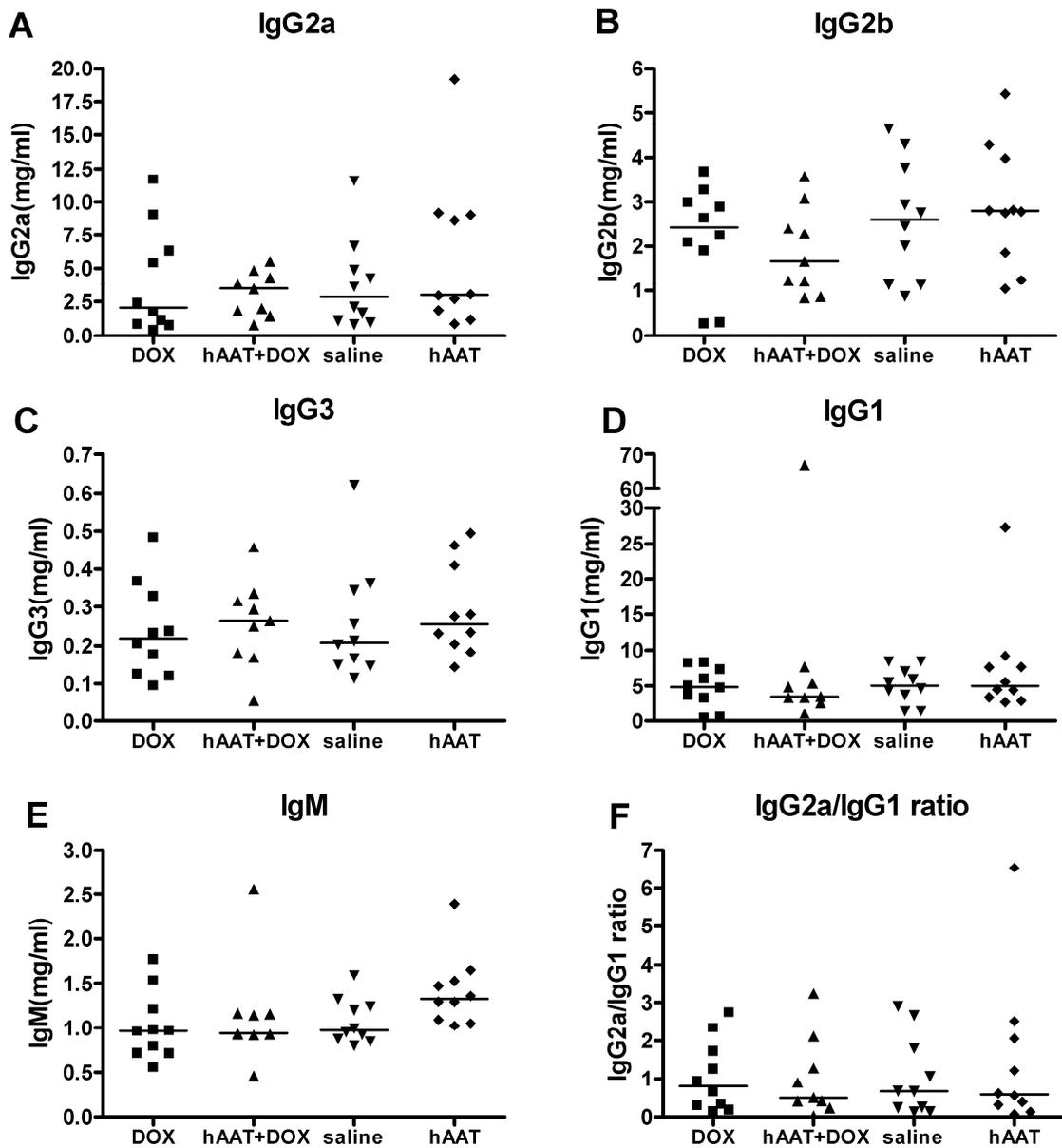


Figure 5-7. Expression of antibody subclasses in pristane treated DBA/1 mice. A, Ig2a antibodies (the predominant isotype in pristane treated mice), B, IgG2b antibodies, C, IgG3 antibodies, D, IgG1 antibodies, E, IgM antibodies, F, ratio of IgG2a/IgG1 antibodies. Total antibody subclass levels were determined in sera 34 weeks after pristane injection. Each dot represents level of an individual mouse as determined by standard ELISA. Horizontal line represents median.

CHAPTER 6 SUMMARY AND PERSPECTIVES

Summary

Therapeutic approaches for the treatment of rheumatoid arthritis changed dramatically within the last two decades. We now know that proinflammatory cytokines play a pivotal role for disease onset and progression. Advances in the field of immunology, molecular biology as well as a better understanding of disease pathogenesis resulted in the development of drugs with anti-inflammatory and immunomodulatory properties such as blockade of TNF- α and IL-1 β as well as inhibition of T-cells and B-cells. Introduction of these biologics significantly changed the standard treatment regimen for RA. Although methotrexate is still a cornerstone in rheumatoid arthritis, today's standard therapy uses the new biologics or combinations with methotrexate. Their introduction showed significant improvement in many patients including those which showed no response to the applied standard therapies with methotrexate (29).

Unfortunately, increasing evidence suggests that these new biologics are not as promising as anticipated. Although most studies showed great efficiency, some long term studies revealed their inefficiency in selected patients and occurrence of severe side effects (21). As mentioned before, it has been shown that anti-TNF- α therapy increased the risk of infections as well as malignancies (27-29). In addition prescribing these drugs for treatment relieves the symptoms and prevents some patients from further joint damage, but they do not fight the actual cause and therefore have to be taken life long, which creates a financial burden for many patients.

Therefore, there is still the need to develop new strategies and drugs for effective treatment. The studies described here are a new and promising approach which may lead to future therapies applicable for rheumatoid arthritis. Human AAT, which is the therapeutic protein we used in our study, has anti-inflammatory properties and its serum level increases 3-4

fold in response to inflammation. It therefore may be a major contributor for suppressing inflammation in the body. Due to its high endogenous concentration, side effects may be limited when applied in high therapeutic concentrations in clinical settings. Our studies and those from other groups also indicate that hAAT has anti-inflammatory properties by not targeting a single cytokine but many with medium efficiency (95). This might be advantageous since total blockade of a specific cytokine can cause severe side effects as seen in commonly applied anti-cytokine therapy for RA.

Current therapies are accompanied with high costs and the requirement of regular injections, reducing patients' compliance. For treatment, the protein has to be administrated in high doses to ensure effective concentrations in target tissues, therefore increasing the risk of unwanted side effects. Our approach of using gene therapy should demonstrate that it is possible to circumvent these regular injections of high protein doses and still ensuring therapeutic protein serum levels. We used the AAV8 vector to deliver the hAAT gene since it has been shown that AAV8 is able to infect various tissues, including muscle and liver, ensuring systemic expression of the delivered protein. This is desirable in a model for rheumatoid arthritis in which multiple joints ubiquitous in the body are affected. Local injection in each single joint would be time consuming and less efficient especially in smaller joints. AAV8 also has a low immunogenicity compared to other AAV serotypes or other viral vectors. Especially when compared with different vectors such as adenovirus or retrovirus the low immunogenicity results in higher and more effective gene expression.

After receiving promising results that hAAT is effective in reducing arthritis development by using protein as well as gene therapy, we intended to further improve and optimize the gene delivery approach. In the initial studies we used a CB-promoter resulting in constant hAAT

expression in the infected cells; even in non-acute phases of the disease in which hAAT might not be needed. As mentioned before, it is desirable to control gene expression in order to limit high therapeutic protein levels to active disease stages. For this purpose we administered AAV8 vector which delivered hAAT gene under control of a tetracycline dependent promoter. Using this expression system not just enables us to control hAAT gene expression but also requires doxycycline administration which has an additional therapeutic effect. In this unique approach, doxycycline is used as a therapeutic agent and to regulate hAAT expression. We were able to show that administration of both hAAT and doxycycline improves the treatment effect compared to monotherapies.

As mentioned before AAV8 mediated gene delivery is characterized by low immunogenicity of the vector. We observed that antibody development against the transgene is almost absent and a high sustained serum level of the therapeutic protein hAAT indicates that an immune response against the vector also seems to be very low. This is certainly an interesting and unexpected observation since previous studies showed that other AAV serotypes such as AAV2 do elicit an immune response against the transgene or the vector (70, 71, 148). In addition we also observed that autoantibodies against collagen II in CIA model are initially reduced after hAAT protein therapy and this effect is even more enhanced and more sustained after gene delivery of hAAT. This observation might reveal the great potential of AAV8-hAAT mediated gene delivery for autoimmune diseases where autoantibodies play a pivotal role. We therefore transferred the gene therapeutic approach to a different arthritis animal model to test the feasibility of hAAT therapy and its effect on autoantibody development. We used pristane induced arthritis mouse model which is characterized by development of inflammatory arthritis accompanied with development of lupus specific autoantibodies. Although we were not able to

observe an anti-arthritic effect in this model we saw a trend that hAAT in combination with doxycycline is able to suppress lupus autoantibody anti-Su. This observation encourages us to believe that AAV8-hAAT maybe a promising agent to suppress autoantibody development in autoimmune diseases.

Perspectives

The studies presented in this dissertation describe alternative approaches to current therapies for rheumatoid arthritis. The observation that promising treatments with new biologics which evolved in the last two decades have shown to cause side effects and also turned out to be inefficient in some patients, emphasizes the needs to further improve and develop new therapy approaches in arthritic diseases. Due to the fact that rheumatoid arthritis is a complex disease where several pathways have to be regulated in order to achieve a sufficient therapeutic effect, monotherapies are often not as successful as combination therapies. The introduction of doxycycline is therefore reasonable since it adds therapeutic potential to the therapy by targeting metalloproteinases in the synovium. The anti-arthritic potential of doxycycline monotherapy in humans seems to be questionable as seen in a study recently published (149). Interestingly, it is able to improve the therapeutic effect when it is combined with methotrexate (108). It would therefore be interesting to see if doxycycline is also able to improve the therapy effect when it is combined with other commonly used drugs in arthritis such as the new biologicals targeting TNF- α or IL-1 β . Our approach to combine doxycycline with hAAT also revealed an improved therapeutic effect. It has still to be elucidated if blocking of MMP's is the only therapeutic effect of doxycycline. Our studies suggest that doxycycline in addition is able to reduce proinflammatory cytokine release such as IL-6 from fibroblasts. As described previously, antibiotics administered in RA patients might also eliminate potential environmental triggers

involved in disease onset suggesting an antimicrobial effect being responsible for disease suppression (150).

When examining current anti-cytokine therapies, the concern of potential side effects in response to a complete blocking of a single cytokine arises. The complexity of cytokine/chemokine network and its integrity in the immune system suggest that it is quite reasonable to expect unwanted consequences. It is therefore reasonable to develop an approach where relevant cytokines are affected but not as much as in current therapies. Further research is required to test the efficiency of arthritis suppression by targeting multiple cytokines in a moderate manner. Our approach, using hAAT might be a first step in this direction since it has been shown that hAAT has moderate immunomodulatory effects by down regulating proinflammatory IL-1 β , TNF- α , IL-8 and up regulating anti-inflammatory IL-10 (94). If this mechanism is indeed responsible for the effects we have seen in the presented studies has still to be elucidated. Further studies also have to reveal to what extent hAAT regulates the affected cytokines.

A critical aspect when delivering proteins is to ensure that they are available at effective concentrations at the side of action. Due to their structure and properties, proteins might elicit an immune response which results in their elimination. In the studies described, we showed that hAAT serum level is elevated after protein and gene therapy. Although they vary using different delivery methods and animal models, they seem to be in therapeutic range since we observed an anti-arthritic effect. Nevertheless it is definitely desirable to determine whether further increase of hAAT serum level would also improve the therapeutic effect.

Different options to increase local hAAT concentrations are available. Concerning protein therapy we just need to inject higher concentrations of hAAT protein e.g. 1mg/ml or 2mg/ml or

even higher doses and determine actual serum level as well as testing for therapeutic effects. The increase of protein concentration using a gene therapy approach would be more challenging. We have to optimize gene delivery by either using a higher vector dose, a different AAV serotype, perform local vector administration or perform ex vivo gene delivery approaches. Increasing the vector dose may be the easiest to perform but it is also a very expensive alternative. In addition we need to determine whether this results in high protein levels without causing noteworthy immune responses.

Using a different serotype is also challenging because efficient infection of target tissues has to be ensured and most so far known AAV vectors do elicit some immune responses which limits transgene delivery. Local vector administration would ensure high vector concentration at the target tissue but as mentioned before since RA is a systemic disease and multiple joints are affected and it is challenging to inject the vector in every single affected joints, including the very small ones.

A certainly interesting approach would be ex vivo gene transfer to cells which are major players in RA pathogenesis and therefore target affected joints. Such cells include macrophages, T-cells and B-cells. They would home to the sides of inflammation and the transgene is expressed locally where it is needed.

To circumvent the obstacles seen in protein therapy such as being time consuming and very costly, gene therapy might be the delivery method of choice for future RA therapy. Despite all the drawbacks seen in the field in the last decade, including malignancies and failed therapeutic efficiency, researchers in the field are still convinced that it has a promising future. Continuous vector improvement and understanding of its biology are certainly necessary to

achieve the proposed goals and ensuring that gene therapy resembles a promising future therapy providing a save, economic, efficient and convenient alternative of drug delivery.

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BIOGRAPHICAL SKETCH

Christian Alexander Grimstein was born in Georgsmarienhütte, Germany in 1978. After graduating from high school in 1998 he performed civilian service at a hospital in Osnabrück, Germany in the Department of Laboratory Medicine. He started pharmacy school at Saarland University in 1999 and received his bachelor's degree in pharmaceutical sciences in 2003. He became Registered Pharmacist in Germany in 2004. After that he started the graduate program in the Department of Pharmaceutics at University of Florida, Gainesville in January 2005. He worked under supervision of Dr. Sihong Song on protein and gene therapy of alpha-1 antitrypsin for experimental rheumatoid arthritis.