Effects of manipulating the immune system on dystrophin gene transfer and dystrophic phenotype in striated muscles of Duchenne muscular dystrophy model, *mdx* mouse

by

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Duchenne muscular dystrophy (DMD) is a fatal genetic disorder caused by mutations in the gene coding for dystrophin protein, which give rise to a dysfunctional protein in skeletal muscle. Dystrophic muscle progressively degenerates. In addition, necrotic muscle fibers undergo high levels of inflammation that in turn promote the pathology that is associated with this devastating disease. Therefore, treatments that 1) restore expression a functional dystrophin protein in dystrophic muscles, and 2) lower the ongoing inflammation in the necrotic muscle tissue, are both important in ameliorating DMD phenotype. Transfer of a functional dystrophin gene using a viral vector can help restore the missing dystrophin protein in dystrophic muscles. The host immune system, however, is a major barrier to successful vector-mediated dystrophin protein expression in a dystrophic host, as anti-dystrophin immune response leads to rejection of the protein. Here I show that temporal elimination of the host immune system by irradiation in the *mdx* mouse, a murine model of DMD, prior to vector-mediated dystrophin gene delivery, leads to a delayed and diminished host anti-dystrophin immune response. These findings are important for a better evaluation of anti-dystrophin immunity in a dystrophic host. In the case of lowering inflammation in dystrophic muscles, I investigated the effects of rapamycin, a potent immunosuppressant, on both dystrophic phenotype and dystrophin gene transfer in *mdx* mice. Treatment of adult *mdx* muscles with rapamycin lead to significantly lower levels of muscle fiber necrosis and reduced effector T cell infiltration in dystrophic muscles. These events correlated with a difference in activation of the mammalian target of rapamycin (mTOR) in the diaphragm

muscle, but not the TA muscle, suggesting a differential regulation of mTOR activation in the two tissues. Rapamycin treatment, however, did not allow for a higher level of vector-mediated dystrophin protein expression in treated muscles. In general, these findings shed more light on the effects of manipulating the immune system in a dystrophic host in terms of both reducing the inflammation that is associated with DMD and reducing anti-dystrophin responses following gene therapy, suggesting that regulation of the immune system is essential in ameliorating DMD.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

DEDICATION

To my dearest parents, Fakhrosadat Safabakhsh and Bijan Eghtesad, for all the sacrifices that they have made for me and for all their spiritual encouragements. I hope to be able to make up even the tiniest amount of their efforts by making them proud today.

To my wonderful husband and my best friend, Alireza Rezaee, for all the true support that he provided for me during the hard times of my work and for tolerating my insanity at times that my data did not look desirable!

To my kindest sisters, Soodeh and Sareh, for always being there for me in practically any way that I needed.

To Dr. Paula R. Clemens, my excellent mentor and adviser, for always believing in me and my ideas, and for teaching me beyond science and laboratory experience.

And

To all DMD patients and their families whose suffer is not describable in words
I hope and pray to have played at least a small role in the process of finding ways to reduce or eliminate their pain.

PREFACE

A famous Persian poem says:

If the Lord closes a door by His wisdom He will open another one by His blessings

As a researcher, I truly experienced this poem during the time that I was pursuing my Ph.D. degree. Meeting the right people at the right time was what God gave me through His beneficence. During my years as a graduate student, there were times when a big part of my work seemed to be moving slowly or in the wrong direction. Fortunately, however, just when I was discouraged, I would meet with someone who would completely change things around for me, helping me re-gain my enthusiasm for my research and find my way. Now, as I write my Ph.D. thesis, I would like to acknowledge those "right people" whom I met at the "right time."

Angela Green. I met with Angie for the first time at a BGSA symposium. I was then a first-year student working on my thesis proposal, desperately trying to find a "theme" for my project. And there were the Tregs in Angie's work! I truly enjoyed learning about them. I mentioned them to Dr. Clemens, and as always, she supported my ideas and that was how Tregs and the idea of down-regulation of the immune system became the theme in my Ph.D. project.

Theresa Whiteside/Laura Strauss: I had just passed a huge gap after losing all of our mice due to an MPV break-out in our animal facility, and was in the middle of another gap, trying endlessly with no apparent success to make a vector that was to be used in Treg expansion

assays. I met with Dr. Whiteside and her then post-doctoral fellow, Laura, at an immunology seminar, as they presented their work on using rapamycin to expand Tregs. I was fortunate enough to be able to spend some time with Laura in their lab, learning more about rapamycin and their assays. Thanks to Dr. Clemens' believing in me, learning about their work helped me add a completely new project to my work that turned out to be a very interesting one.

Siddharth Jhunjhunwala: As the *in vitro* rapamycin studies were moving slowly, the *in vivo* work, which was the ultimate goal, seemed to be tougher that I had expected, as a very high number of Tregs had to be prepared and injected into the mice. I had trouble finding out how to control these Tregs to enter the muscle tissue; and there I was at the University of Pittsburgh Science 2008 event, chatting with my good friend, Rakshita, who introduced me to Sid. This time Sid, a graduate student working with rapamycin microparticles to deliver rapamycin *in vivo* locally, helped me with move on in my work. We talked for some time about our projects and shortly after mentioning it to Dr. Clemens, we became collaborators. That event extremely affected the speed of my work and added an interesting section to my project.

It is needless to say that many more people have helped me and affected my work to make it possible for me to be where I am today. The people mentioned here are those who provided turning-points in my experience, making the poem mentioned at the beginning of this section more meaningful for me than ever.

STATEMENT OF PROBLEM

Muscular dystrophies refer to a group of genetic disorders that are associated with progressive muscle weakness and wasting. These diseases are due to one or more muscle protein defects and in general affect more than just mobility; often leading to destruction of cardiac, nervous, and respiratory systems as well, the latter being a result of muscle wasting in diaphragm tissue. An example of a common and severe muscular dystrophy that ultimately leads to an early death in patients is Duchenne muscular dystrophy (DMD), affecting 1 in 3,600-6,000 live male births per year [1-3]. DMD is due to mutations in the X-linked gene coding for dystrophin protein, which is an essential muscle structural protein.

For years gene therapy has been considered a potential way to cure DMD. Transfer of the large full-length dystrophin cDNA of about 11 kb has become possible due to generation of high capacity adenoviral (HC-Ad) vectors, which are depleted of all viral genes [4-6]. Despite the advantages of this vector over the previously used adenoviral vectors, the host immune response to the vector and the therapeutic gene product is still considered a major barrier to successful dystrophin gene transfer [7-9].

Several approaches to overcoming the problem of vector and gene product rejection by the host immune system have been tried, including transient blockage of immune system stimulation at the time of gene delivery [10], as well as delivering the vector before full maturation of host immune system (in the case of mice) [11-15]. Nonetheless, the host's humoral and cellular

immune responses against the therapeutic gene product, which ultimately reduce, if not abolish, therapeutic gene expression, have not been fully eliminated. It is thus crucial to understand the role of the host immune system in abolishing vector-mediated dystrophin protein expression in dystrophic muscles.

In addition to the host immune response to transferred gene product, untreated dystrophic muscle tissue is associated with immunological complications, such as massive infiltration of T cells that promote the pathology of the disease [16-18]. Therefore, besides the genetic mutations that lead to the pathogenicity of DMD, auto-immune-like reactions in the damaged muscles are important factors in disease progression. Thus, it is important to better understand the role and pattern of immune responses in dystrophic muscles due to the disease and to test different approaches to ameliorate dystrophic pathology.

The subsequent pages describe studies that were performed to 1) eliminate and understand host anti-dystrophin immune responses to HC-Ad vector-mediated dystrophin cDNA transfer in dystrophic *mdx* mouse; 2) examine the effect of temporal local and systemic immune suppression on dystrophic phenotype of adult *mdx* mice; 3) assess the possibility of using local immune suppression for eliminating host immune response against vector-mediated dystrophin expression; and 4) evaluate the effect of muscle injury on immunity in muscle tissue. These studies ultimately enhance our knowledge of immune reactions in dystrophic *mdx* mice to both foreign and self-antigens, leading to approaches that may one day contribute toward a cure for DMD.

1.0 INTRODUCTION

 Part of the following section has been published: Immunology. 2009 May;127(1):1-7. and Ann Neurol. 2009 Sep;66(3):267-70.

1.1 DUCHENNE MUSCULAR DYSTROPHY

The most common type of muscular dystrophies is Duchenne muscular dystrophy (DMD), which is an X-linked recessive muscle-degenerative disorder caused by mutations, mostly deletions, in the largest known gene, which codes for dystrophin. Dystrophin protein has a molecular weight of about 427 kb [19-21] and carries out an essential structural role in muscle fibers. Dystrophin physically links the cytoskeleton, more specifically actin filaments, in muscle fibers to the extra-cellular matrix or basal lamina through its interactions with the membrane-bound dystroglycan complex that is in turn connected to laminin in the basal lamina (Fig. 1). Lack of a functional dystrophin protein gives rise to an unstable muscle fiber membrane or sarcolemma that becomes extremely susceptible to damage due to muscle contraction.

The unstable muscle membrane becomes “leaky” and allows for molecules travelling in and out of the muscle fibers; an event that can lead to necrosis of the fibers. Examples of molecules that can enter the cell are calcium ions (Ca^{++}) that enter the negatively charged muscle

membrane and upon cell entry can lead to an overload of Ca^{++} in the muscle fiber cytoplasm, leading to cell death through necrosis [22-25]. In general, an imbalance in Ca^{++} homeostasis has been linked to many pathological features in different tissues, including muscle, [25, 26] and this mechanism of muscle fiber damage has been considered a major one in DMD.

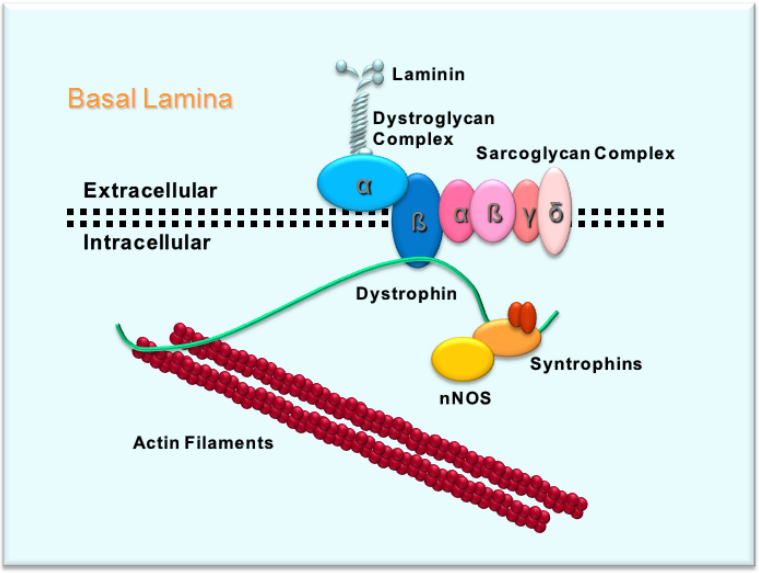

Figure 1: Schematic of the dystrophin-associated proteins at the sarcolemma. Dystrophin and its associated proteins have important roles in maintaining muscle membrane or sarcolemma stability. Dystrophin protein, which is a crucial structural protein in muscle fibers links actin filaments to the basal lamina through its interactions with the membrane-bound dystroglycan and sarcoglycan complexes. Absence of almost any of the complex-associated proteins can lead to an unstable membrane leading to muscular dystrophy.

Duchenne muscular dystrophy affects approximately 1 in 3600-6000 live male births per year [1-3]. DMD patients are usually diagnosed at around 3-5 years of age, which is when their physical characteristics and mobility becomes noticeably different from their peers. Parents of DMD patients may notice manifestations of muscle weakness, such as delayed walking, frequent falls, and difficulty in running, jumping, or climbing stairs. Affected boys have increased lumber lordosis and end up having spinal curvatures that can become very painful and sometimes need surgery to resolve this problem. In addition, these patients normally have a hard time standing up from a sitting position and normally need to push their arms against the ground or their knees in order to be able to stand up, a common symptom known as the Gowers' maneuver.[27] Aside from the physical symptoms there are other clinical symptoms associated with DMD including high serum levels of creatine kinase, which is a muscle-specific protein that leaks out of damaged

muscle fiber sarcolemma into serum [28]. In addition, muscle biopsies of DMD patients, as in other muscular dystrophies, indicate ongoing muscle fiber necrosis, replacement of muscle tissue with fibrotic and fatty tissue, and infiltration of inflammatory cells in the muscle tissue.

As the disease progresses these boys lose mobility almost completely and become wheelchair-dependent for movement by 10 to 12 years of age. Although inevitable, this loss of function can sometimes be delayed by chronic treatment with corticosteroid medications. Many patients require respirators in their late teen years since their diaphragms become fibrotic and too weak to perform normal respiration. DMD is a lethal disorder and generally leads to an early death of the patients in the third decade of their lives, usually due to respiratory and cardiac failure.

1.2 IMMUNOLOGICAL COMPLICATIONS ASSOCIATED WITH DMD

DMD is primarily a genetic disorder that gives rise to unstable muscle fiber membranes and leads to muscle wasting. Nonetheless, high numbers of infiltrating cells, including T cells, are also observed in the dystrophic tissues (Fig. 2), indicating that these cells may contribute to DMD-associated pathology. In fact, in recent years different studies have suggested an important role for the immune system in a dystrophic patient or animal models for actually promoting muscle damage. [18] One of the most common animal models to be used in DMD studies is the murine model, the *mdx* mouse, which is a naturally mutated dystrophic model.[29-33] The *mdx* mouse, similar to DMD patients, lacks expression of a functional dystrophin protein in its skeletal muscle fibers due to a point mutation in the dystrophin gene on its X chromosome,

giving rise to a premature stop codon.[33] Similar to DMD patients, pathology of the *mdx* mouse skeletal muscle is also associated with inflammation. The observed massive infiltration of immune cells, including T cell subsets, to the damaged muscle tissue in an untreated DMD patient or the *mdx* mouse is expected considering the high level of ongoing necrotic events in muscle tissues that lead to exposure of all muscle antigens in abnormal levels.

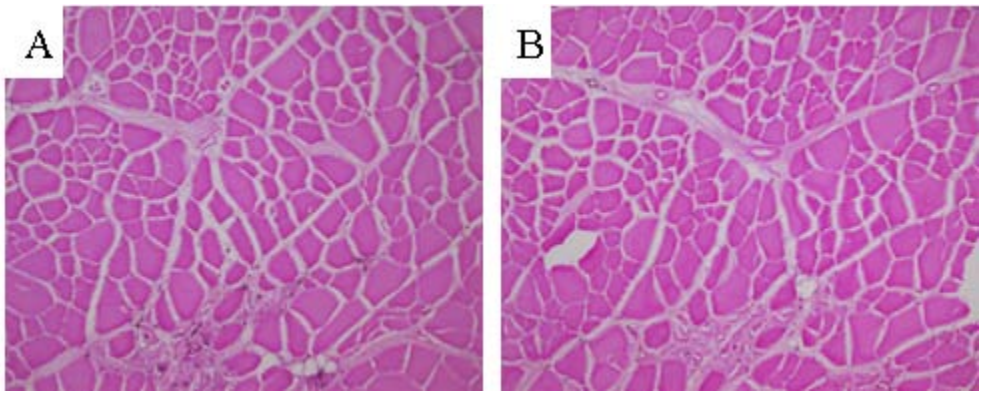

Figure 2: T cell infiltration in dystrophic *mdx* mouse muscles. Infiltrating CD4+ (A) and CD8+ (B) T cells are spread among damaged muscle fibers in DMD patients and damaged muscle fibers in DMD patients and *mdx* mouse.

However, the fact that depletion of $CD4^+$ and $CD8^+$ T cells or even B cells from the *mdx* mouse system leads to a significant decrease in disease-associated muscle pathology [16-18] indicates that the loss of muscle mass may not only be a consequence of the defect in dystrophin gene. Although it has not been specifically shown experimentally, it is increasingly believed that the pathology associated with DMD may also be a result of host's immune reaction against muscle antigens that tend to be exposed at much higher levels than normal, leading to severe immune reaction and inflammation in the damaged tissue. Therefore, DMD has progressively been considered a disorder that is due to a combination of a genetic defect and immunological complications. For this reason, not only induction of expression of a functional dystrophin protein in dystrophic muscle should be considered for treating DMD, but different approaches for down-regulating immune cell infiltration into dystrophic muscle tissue should be taken into serious consideration when treatments are studied and designed for DMD patients.

1.3 C. CURRENT TREATMENTS FOR DMD

Although a complete therapy that can cure DMD is not yet available, there are treatments that are used to ameliorate the conditions of DMD patients during their relatively short life time. Such treatments include drug therapy, using corticosteroids (prednisone, for instance) to slow progression of muscle degeneration [34-37] and to lower inflammation in dystrophic muscle. Other treatments also exist that mainly improve the quality of life of DMD patients that include using assistive devices such as braces and wheelchairs to help patients with their movement and mobility, and sometimes using surgical procedures to treat contractures and spinal curvature that can become severely painful if not treated.

Prednisone is usually given to DMD patients starting at early stages of their disease in order to minimize pathological phenotype associated with the disease. It is important to remember, however, that medications such as prednisone predominantly treat the pathological effects of muscular dystrophy rather than the cause of the disease. One important advantage of such treatment is that it can be done orally and its effect extends systemically to all muscle tissues throughout the body. Side effects of drug treatments may limit their potential benefits, however. For example, prednisone and similar drugs are hormones that can temporarily improve the lives of DMD patients, but in the long term have side effects [38], including interfering with secretion of other hormones needed. Therefore, this treatment, which happens to be one of the most common treatments available at this point, may not be fully beneficial for the patients. The assistive devices available for DMD patients include braces for weak arms and legs that are used for standing up or walking and can help affected boys stay mobile for a slightly longer period of time, wheelchairs that are for middle stages of the disease when patients lose ability to walk

completely, and ventilators that are used at later stages of the disease when patients have had a severe progression of the disease and are no longer able to breathe normally. Finally, there are also some selective surgical treatments that may improve the spine problems of DMD patients, normally performed at different stages of the disease. [39, 40] However, at this point there is no cure for DMD and extensive studies are required to find a way to perhaps restore a functional dystrophin gene in muscle tissues of these patients.

1.4 ONGOING RESEARCH FOR DMD TREATMENT

In addition to the treatments mentioned above that are solely to improve the quality of the short lives of DMD patients by slowing down disease progression, there are also treatments that are under investigation and some in clinical trials. These treatments are mostly evaluated as potential cures for DMD and include cell and gene therapy.

Cell therapy for DMD is a cell-mediated dystrophin expression in muscles of a dystrophic host. In the case of cell therapy in dystrophic muscle tissue, there are muscle stem cells, also referred to as satellite cells or myoblasts, from healthy donors that produce normal dystrophin protein that can be injected into muscle tissue with defective dystrophin gene, as reviewed by Farini *et. al.* [41] These cells have the potential to fuse with the damaged muscle tissue and therefore give rise to muscle fibers that contain a full-length dystrophin gene and produce a functional dystrophin protein, as the nuclei of donor cells contain a healthy dystrophin gene. Fusion of donor cells and dystrophic muscle fibers has been shown in several studies. In one such study, after transfer of muscle stem cells into *mdx* mouse, they found fused fibers that

showed two distinct muscle types with either skeletal muscle characteristics or cardiac muscle characteristics,[42] indicating that cell therapy indeed has a potential therapeutic effect on correcting both skeletal and cardiac muscle tissue in a dystrophic host. In addition to studies in which cells from healthy donors were transferred into dystrophic muscle tissue, there are have also been studies in which myoblasts from dystrophic donors have been genetically manipulated *ex vivo* to induce dystrophin expression in those cells prior to transferring them into a dystrophic mouse or human host.[43, 44]

Although these approaches mentioned above have shown promise in the field of cell therapy for DMD, there are various challenges that will need to be addressed in the future studies in this field in order to be able to consider cell therapy as a successful treatment for DMD. One problem with cell therapy is its efficiency, as the donor cells usually need to be directly injected nto the damaged muscle tissue and they do not travel very far from the injection sites. Therefore, high numbers of injections are required for each muscle to be treated. Researchers are currently examining ways to improve the efficiency of injections. In addition to these technical issues, there are also obstacles at the cellular level, including the life-span of the transferred cells. As demonstrated in one study, transferred cells may only last a few hours in the dystrophic host.[45] The rapid death of transferred cells may, as one study suggests, be due to the infiltration of host T cells as part of the adaptive immunity.[46] It has been shown, however, that the innate immune system may not affect transferred cells in dystrophic muscle tissue.[47] In general, the host immune system presents a significant barrier to successful cell transplantation, similar to organ or tissue transplantation. Various studies have addressed the problem of immune response against transferred cells expressing the dystrophin protein in dystrophic hosts using immunosuppressive drugs such as FK506[48] or Cyclophosphamide. [49] Although immunosuppression is required for

preventing immune system-mediated rejection of the transplanted cells, factors such as toxicity of the immunosuppressive agent may have negative effects on the host, as was suggested when Cyclophosphamide was used in *mdx* mice undergoing myoblast transplantation.[49] To eliminate host immunity against transferred cells, irradiation of the host has also been studied, which has not been very successful as the cells survived for a short period of time.[50] In general, although transferring healthy cells with full-length dystrophin gene into dystrophic muscle tissue is a promising approach, there are still many obstacles to overcome before this approach can be considered a practical therapy for DMD.

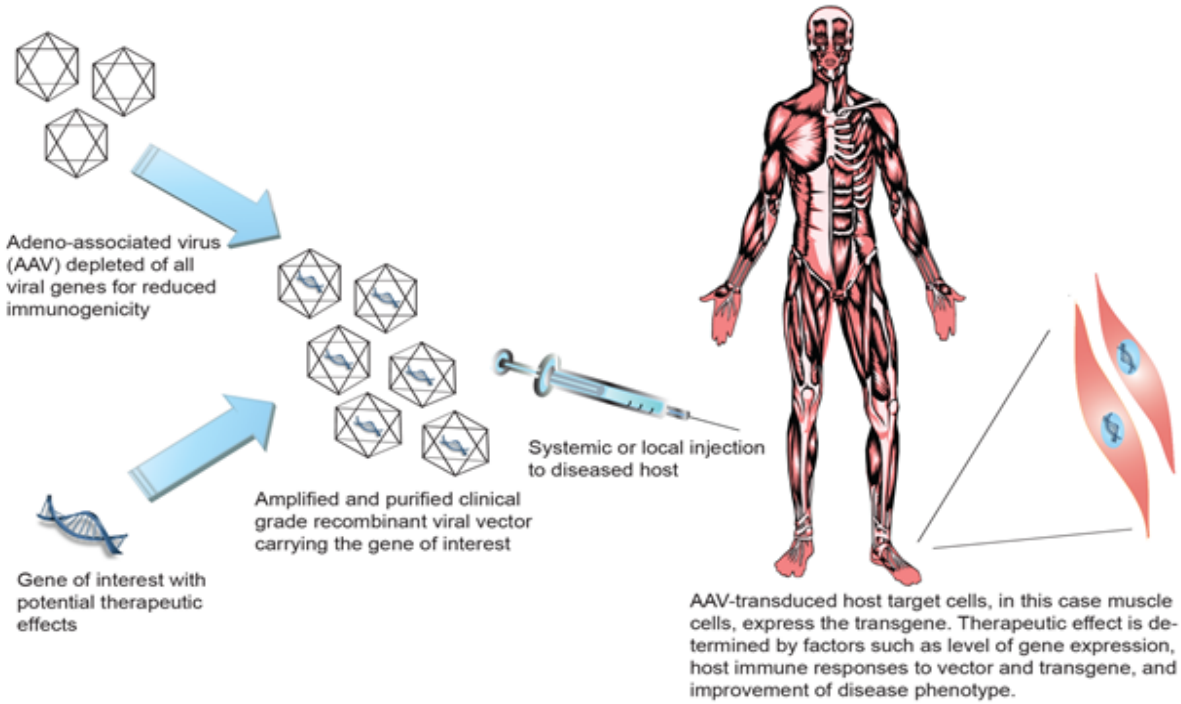

Figure 3: Schematic of viral vector delivery to muscle tissue in humans.

Similar to cell therapy, gene therapy is an approach by which a healthy dystrophin gene may be inserted into a viral vector rather than a cell, and can then be transferred into muscle

fibers and ultimately nuclei of dystrophic muscle tissue (Fig. 3) and therefore can lead to production of a functional dystrophin protein in a dystrophic host. For this purpose, the most common means by which the dystrophin gene is transferred into damaged muscle tissue is using viral vectors that can infect muscle fibers and transfer the gene of interest into the target tissue. Amongst the challenges that are faced in studies on gene therapy for DMD are efficiency of gene delivery to target fibers and the host immune system that may lead to rejection of the viral vector and the transferred gene. The latter problem has been shown to be to some degree dependent on the type of viral vector used and the organism on which the studies are conducted. In the following section the gene therapy approach will be discussed in more detail, as the majority of subsequent studies explained here deal with dystrophin gene transfer in dystrophic hosts.

1.5 POTENTIAL APPROACHES FOR A CURE: GENE THERAPY

Many researchers worldwide study potential ways to cure DMD. As with any other loss-of-function genetic disorder with a single-gene mutation, one potential approach to treat DMD is therapeutic gene replacement that gives rise to expression of a functional dystrophin protein in dystrophic muscle fibers. Dystrophin protein is a large protein with a cDNA of about 14 kb. Therefore, it is important to use the vector system with a capacity for this long DNA insert.

Some groups have suggested using a shorter version of the dystrophin cDNA to only restore a short or “mini-dystrophin” in the treated dystrophic muscle tissues. [51, 52] One of the most common vector systems to be used to transfer this mini-dystrophin is the adeno-associated

viral (AAV) vector system, which is derived from a small virus from the Parvavirus family and has a capacity for a DNA insert of less than 5kb.[53] Although expanding packaging capacity is being considered, [54-56] at this point the low capacity of AAV will allow for only a highly truncated dystrophin cDNA to be transferred into the damaged muscle tissue.

One of the most considerable advantages associated with the AAV system is the fact that these viral vectors do not seem to be pathogenic in humans. There are still many questions that remain with regard to the immunological responses of the host against these vectors, as some contradictory data on their immunogenicity is available at this point.[57-60] Also, although successful transgene expression has been achieved with mini-dystrophin in the mouse *mdx* model, it is not yet clear if this small dystrophin protein will be functional in DMD dystrophic muscle. Of note, similar truncated versions of dystrophin cause the allelic form of muscular dystrophy known as Becker's muscular dystrophy (BMD). Thus, providing conditions in which a full-length dystrophin protein may be transferred into the host system is important.

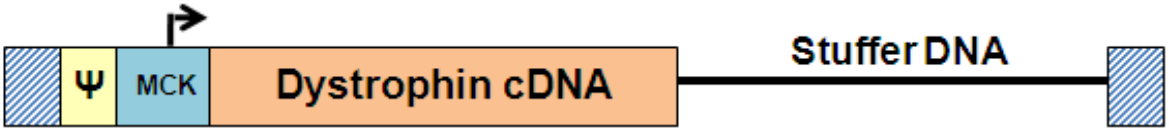

Figure 4: Schematic of HC-Ad vector carrying dystrophin cDNA. Depletion of all viral genes from adenoviral vector enables scientists to insert a double-stranded DNA of about 30 kb. This will allow for a full-length dystrophin cDNA to be inserted into the vector.

A generation of adenoviral vectors referred to as high-capacity adenoviral (HC-Ad) vectors, which are depleted of all viral genes and have a DNA-carrying capacity of approximately 30 kb[61] can be used to transfer this large cDNA (Fig. 4), as has been shown in previous work [5, 6, 9, 10, 62].

The advantage of this vector over the previous generations of adenoviral vector, including first and second generation, in which only segments of viral genes were replaced with the DNA insert, is that 1) using this vector system a full-length dystrophin can be expressed in the target tissue, whereas with the previous generations a truncated version of dystrophin protein was provided to the host, and 2) due to the lack of viral genes, which if present, could allow for production of viral proteins in the host, this vector seems to be less immunogenic, as it transfers fewer viral antigens. These two characteristics of the HC-Ad vectors make them one of the better options available for dystrophin gene therapy. Nonetheless, since it is necessary to use adenoviral viruses, referred to as helper viruses, that contain viral genes required for packaging of the HC-Ad vectors,[63] the final HC-Ad vector product may contain some contaminating helper-viruses that are capable of producing viral proteins in the host system.

One important factor affecting the efficiency of vector-mediated dystrophin expression is the promoter that controls transgene expression in the desired tissue. When targeting a specific tissue or organ in the host, it is crucial to use a tissue-specific promoter, such as the muscle creatine kinase (MCK) promoter for muscle cells (Fig. 4), that can result in specific expression of the transferred gene only in the desired tissue or organ. Using a tissue-specific promoter will prevent potential side effects of expressing a gene in irrelevant tissues and will lower the immunogenicity of the transferred gene product, as it is less exposed to the host immune system. It is particularly important to control specific tissue targeting and transgene expression because it has been shown in various studies that expression of a transgene in organs such as liver, when liver is not the target tissue, may lead to high levels of host immune response against the transgene product, as has been reviewed by Di Paolo and Shayakhmetov.[64]

In general, further studies to lower the host immunological reactions against transgene product, taking advantage of vectors such as HC-Ad vector that are less immunogenic compared to previous generations of adenoviral vectors and using techniques that lead to tissue-specific transgene expression are important for successful therapeutic gene delivery. Previous work has been done using the HC-Ad vectors to transfer a single or even two copies of full-length dystrophin in *mdx* mouse muscle tissue [5]. Even though the vector system used seems to be less immunogenic compared to some previously used vectors, expression of dystrophin protein in these studies still is not completely successful in the host system, as immunological complications interfere with expression of the transgene at a desired rate and for a long period of time following vector delivery.

1.6 OBSTACLES TO GENE THERAPY: IMMUNOLOGICAL COMPLICATIONS

Strategies of gene transfer must contend with the risk of a host immune reaction against the products encoded by the transferred gene. Studies on gene therapy for genetic diseases such as hemophilia B or DMD, where a gene is either defective or missing, have revealed that expression of the therapeutic gene product may be hampered by the host immune system. This occurs because the normal mechanisms of self tolerance, such as auto-reactive T cell deletion in the thymus during T cell development,[65] cannot occur if the self protein is absent or mutated. As a result, helper $CD4^+$ T cells may develop that recognize the transferred gene product when it is presented by professional antigen presenting cells known as dendritic cells (DCs), and become activated. In some cases, particularly when the therapeutic gene product is a soluble protein,

these activated T cells can then induce B cell activation and as a consequence neutralizing antibodies against the introduced protein are formed, as reviewed by Tarlinton and colleagues,[66] leading to inactivation of its therapeutic role. In addition, the helper T cells can provide signals for activation of cytotoxic $CD8^+$ T cells that in turn can induce killing of the cells expressing the transferred gene product. Robust activation of the adaptive immune response requires the presentation of the foreign protein in the context of an inflammatory response usually mediated by the innate immune system. Thus the choice of vector for gene therapy becomes very important since DNA and RNA motifs from viral vectors can activate cells of the innate immune system, leading to more effective presentation of the gene product to T and B cells.

With gene therapy, the induction of a host immune response significantly depends on factors such as the type of vector used for gene delivery,[67-70] the target tissue,[71] and the route of gene transfer.[72-74] Therefore, in a variety of different gene replacement strategies, variable types and degrees of immunological response to vector particles and/or transgene product are observed that primarily lead to the loss of transgene expression. In response, one approach has been to use less immunogenic vectors or to employ specific routes of delivery that lead to more effective gene replacement, at least in preclinical models. An example of such an approach is the use of the AAV vector for administration of the factor IX gene to hepatocytes of hemophilia B animal models, where sustained gene expression, as well as immune tolerance to the transferred gene was observed.[75, 76] More often, however, high levels of target tissue inflammation are observed after gene transfer, with consequent elimination of the gene product.

Furthermore, even if a preclinical study was successful, the results of subsequent clinical trials do not necessarily mirror what was found in animal studies. A recent example comes from the clinical trials on hemophilia B, where an AAV vector carrying the factor IX gene was

introduced by hepatic gene transfer to participants enrolled in a human study. In this study, despite successful pre-clinical animal studies in mice and dogs,[76-79] initial human clinical studies encountered immunologic challenges. Following gene transfer, elevated liver enzymes were observed in these patients, indicating rejection of their transduced hepatocytes, likely due to re-activation of pre-existing anti-AAV-capsid $CD8^+$ T cells in the recipients.[58] Such an outcome highlights the importance of generating animal models that better resemble human subjects.

To achieve the therapeutic goals of gene delivery into a host with a genetic disorder, modification of the host immune system to completely accept the transgene product is necessary. Various manipulations of the immune system have been attempted in order to minimize the immune recognition of transferred gene products to allow for prolonged gene product expression.[6, 10] One promising approach is to encourage the host immune system to suppress undesirable gene product-specific immune responses by stimulating the generation of regulatory T (Treg) cells. Similar strategies have been used in studies on autoimmune diseases, including type I diabetes, where Tregs have been either generated or stimulated to prevent the undesired anti-self immunological responses.[80] In fact, considering the link between the unwanted anti-self responses in autoimmunity, the responses against a graft in organ transplantation, and the responses against a gene product in the case of therapeutic gene transfer, one could compare studies in each of these fields to better understand the role of Tregs in down-regulating undesirable immune reactions.

1.7 IMMUNE TOLERANCE TO SELF- AND NON-SELF ANTIGENS: REGULATORY T CELLS

Tregs are a remarkable and yet not fully characterized T cell subset in the mammalian immune system, with a crucial role in controlling specific immune responses, to self as well as to foreign antigens. The adaptive immune system in higher vertebrates is capable of reacting to countless non-self antigens. In addition, it is known that auto-reactive T cells survive thymic negative selection; the process by which T cells that recognize self-antigens with high avidity are eliminated. Such auto-reactive T cells have the potential to cause autoimmune disease.

Moreover, a very strong and prolonged reaction to a pathogen may be dangerous to the host [81], since this can lead to massive inflammation that can seriously damage the involved tissue. Tregs play an essential role in controlling desired immune reactions and preventing unwanted autoimmune responses. In fact, the critical role for Tregs in the prevention of autoimmunity is best illustrated by the elimination of Tregs in various animal models.[82, 83] In Scurfy mice, which have a defective foxp3/scurfin gene and therefore lack Tregs,[84] for instance, a massive lympho-proliferative syndrome is observed, where auto-reactive T cells proliferate indefinitely leading to overwhelming autoimmunity.[83-85]

Treg sub-populations, including natural and adaptive Tregs, are important in maintaining peripheral immunological tolerance, as reviewed by Del.[86] Natural Tregs primarily utilize direct or cell-to-cell[87] interactions with other cells of the immune system, while adaptive Tregs utilize indirect or cytokine-mediated[87, 88] interactions. Such interactions are mainly with DCs and activated conventional (helper or cytotoxic) T cells, as reviewed by Vignali *et al.*[89] Interactions of Tregs with DCs through a surface molecule known as LAG-3,[90] as well as the effects of the secreted products of Tregs such as TGF-ß, IL-10, and IL-35[91] on responder T cells are largely

responsible for the immunosuppressive role of Tregs. These direct and/or indirect contacts lead to activation and proliferation of Tregs and consequently to inactivation of responder T cells. The ability of Tregs to down-regulate immunity, which is solely based on the molecular characteristics associated with these cells, renders them as promising candidates for the treatment of autoimmune diseases,[80, 92] and also for the inhibition of inflammatory reactions against a transplanted organ,[93-95] or a transferred therapeutic gene.

Molecular Features: In the two Treg sub-populations mentioned above, natural Tregs arise in the thymus as a consequence of T cell development, and adaptive Tregs arise in the periphery in response to both self and foreign antigens. Natural Tregs are thought to arise as a result of partial negative selection in which self reactive T cells are not deleted, but rather differentiate into Tregs. Adaptive Tregs can be induced in the periphery by the cytokines TGF-β and/or IL-10, or by interaction with peripheral immature DCs expressing low levels of self proteins.[96] As a result of this diversity there is no single cell surface marker that can be used to define Tregs. In addition, many of the markers associated with Tregs can also be found on activated responder $CD4^+$ T cells. To date, a standard approach of most laboratories has been to use a combination of different characteristic markers that together are able to specifically identify Treg cells efficiently. In addition to the CD4 molecule, these markers include the α subunit of interleukin-2 (IL-2) receptor (CD25) in higher levels than on the activated responder T cells, cytotoxic T lymphocyte antigen-4 (CTLA-4), membrane-bound transforming growth factor-ß (TGF-ß), L-selectin (CD62L), glucocorticoid-induced TNF-related receptor (GITR), lymphocyte activated gene-3 (LAG-3), neuropilin-1 (Nrp1),[97-99] Galectin-10 (Gal-10),[100] and the transcription factor forkhead box p3 (Foxp3). Foxp3 seems to be mostly specific to the Treg population. Although recent studies have reported transient expression of Foxp3 in activated

human T cells,[101] this has not been seen in mouse studies. Recently it has also been suggested that high expression levels of the cell surface molecule Folate Receptor 4 (FR4) on T cells could be used to separate Tregs from activated effector T cells in certain conditions after T cell stimulation.[102]

Each of the molecules mentioned is important in the function of Treg cells in down-regulating an immune reaction. For instance, the CTLA-4 molecule interacts with CD80 and CD86 molecules on DC and inhibits the ability of these important co-stimulatory molecules to bind to CD28 on CD4 T cells that is crucial for T cell activation. TGF-ß is an example of immunosuppressive molecules and its signaling along with the help of IL-2 leads to Foxp3 expression in Tregs.[103-105] CD62L is important for the homing of Tregs to the lymph nodes.[106] LAG-3 is a CD4-related molecule with the potential to bind the MHC II molecule on DCs;[107, 108] and Nrp1 is a TGF-beta receptor on the cell surface of Tregs and is suggested to enhance Treg-DC interaction,[97, 99] which are crucial in induction of immunological tolerance. Gal-10 is a lectin family member and has been shown to be constitutively expressed in Tregs, at least in humans, and has been shown to be essential for the suppressive activity of human-derived Tregs.[100] Foxp3 is a transcription factor that is crucial for the suppression of IL-2 expression, as well as up-regulation of Treg-associated proteins such as CD25 and CTLA-4.[109] In addition to the marker molecules that are used to track and/or identify Tregs, there are other markers, such as secreted TGF-ß, IL-10, and IL-35[91, 110] in the cell culture media that have been used in Treg functional assays, in which generally Treg-mediated suppression of responder T cell activity is tested. Together, these different molecules make the ongoing research on Tregs in clinically-relevant scenarios such as autoimmunity, transplantation, cancer, and therapeutic gene replacement, possible.

Because Tregs normally occupy only a small portion of the $CD4^+$ T cell pool, it is important to find ways to increase Treg number and effect. Positive effects of the immunosuppressive drug, rapamycin (RAPA), have been shown on expansion of $Foxp3^+$ Treg population,[111, 112] both *in vitro* and *in vivo*. Aside from its effects on Tregs, RAPA has been shown to affect cell growth and metabolism through its interactions with its target protein known as the target of rapamycin (TOR),[113, 114] which was first characterized in yeast,[115, 116] but it has also been studied in other eukaryotes, including mammals. In mammals, the protein is named mammalian TOR (mTOR). In yeast TOR has been shown to be involved in regulating phosphorylation of ribosomal subunits, and therefore, important in regulating protein synthesis.[117] It has also been studied in terms of the cell cycle process, in which TOR has been shown to be a crucial controlling factor.[118] In mammals, mTOR regulates similar processes in the cell and is currently under investigation. The importance of mTOR in cell growth suggests that it may play a role in dystrophic muscles of *mdx* mice that are undergoing regeneration. Learning about the effects of RAPA on both immune system and pathological features associated with DMD, as well as understanding mTOR activation in dystrophic muscles has not been previously investigated.

Overall, considering the devastating effects of the immune system on therapeutic gene replacement, and on the pathology associated with DMD, it is crucial to understand how immune system manipulations may change both host anti-dystrophin immunity and DMD pathology. In the following pages, three studies are presented to:

1) Better understand the anti-dystrophin immunity in dystrophic mice following vector-mediated dystrophin expression in *mdx* skeletal muscles.

2) Examine the effects of down regulating the immune system and manipulating mTOR activity on dystrophic pathology using the drug RAPA.

3) Test the hypothesis that RAPA-induced immune suppression may lead to a long-term vector-mediated dystrophin expression in *mdx* muscles.

In the first study, I have hypothesized that temporal elimination of host immune cells by irradiation prior to dystrophin gene transfer leads to a delayed immune response and long-term vector-mediated dystrophin protein expression in dystrophic muscles of the *mdx* mouse.

In the second study, my hypothesis was that treating the *mdx* mouse with the immunosuppressive drug, rapamycin, will ameliorate the dystrophic muscle pathology by lowering inflammation and altering mTOR activation.

In the third study, I hypothesized that treatment of the *mdx* mouse with rapamycin prior to dystrophin gene transfer will lead to a lower anti-dystrophin immunity and long-term vector-mediated dystrophin protein expression.

2.0 HOST IMMUNOLOGICAL TOLERANCE TO HC-AD VECTOR-MEDIATED DYSTROPHIN CDNA TRANSFER BY HOST IRRADIATION

2.1 RATIONALE

The following study was conducted to test the hypothesis that an absence of the host immune system in the *mdx* mouse at the time of intramuscular HC-Ad vector-mediated dystrophin cDNA transfer will allow for the returning immune cells to recognize the newly expressed dystrophin protein as a "self" antigen, thus diminishing, if not completely preventing, anti-dystrophin host immune responses leading to successful long-term expression of vector-mediated dystrophin protein expression.

2.2 INTRODUCTION

Previous studies have found that anti-dystrophin antibodies were induced by HC-Ad vector-mediated dystrophin cDNA delivery to muscles of adult *mdx* mice as early as two weeks post-gene transfer.[9] This anti-dystrophin immunity is detected even though rare dystrophin-expressing fibers, known as revertant fibers, are found in *mdx* muscle.[7-9, 119-122] The immune

response of a dystrophic host against the transferred dystrophin gene appears to have contributions from both humoral and cellular reactions against the vector-mediated expressed dystrophin protein.

The response of the host immune system to the transferred gene reflects the normal function of the host defense system against neo-antigens. Because *mdx* mice do not produce dystrophin protein at normal levels, dystrophin may be regarded as a "foreign" antigen when it is expressed at higher levels than background in these dystrophic mice. Very little is known about the anti-dystrophin immune response raised by dystrophin-deficient recipients to dystrophin gene transfer. Therefore, I investigated the anti-dystrophin immune response by manipulating the immune system of adult *mdx* mice through a temporal removal of immune cells prior to vector-mediated murine dystrophin gene delivery. I utilized complementary approaches to temporarily remove the host immune system before gene transfer. First, a low dose of irradiation to temporally deplete only the peripheral immune cells was followed by self-reconstitution of the host's peripheral immune cells after gene transfer. Second, a high dose of irradiation was followed by reconstitution of the central and peripheral immune system with bone marrow (BM) transfer from a syngeneic wild-type donor after gene transfer. The low and high dose irradiation allowed me to explore the relative contributions of the peripheral and central components of the immune response to recombinant murine dystrophin.

In addition to using both low and high doses of irradiation, I took advantage of a HC-Ad vector that enabled me to study dystrophic host immune responses to a full-length dystrophin protein. This was important because when using a mini-dystrophin, for instance, potentially immunogenic epitopes have been removed and a complete study of host immune response to dystrophin protein may not be achieved.

Whole BM from wild-type mice that express endogenous dystrophin protein in striated muscle comprises cells that should be fully tolerant to dystrophin protein. By reconstituting the peripheral, or peripheral and central immune system after intramuscular dystrophin vector transfer, I determined whether the returning or new host immune cells, respectively, recognize the full-length murine dystrophin protein as a self-protein. Amongst important players in recognizing "self" versus "foreign" antigens in the immune system are Treg cells.[82, 83, 85, 123, 124] I further explored the role of Treg cells in the peripheral and central immune response to recombinant, murine dystrophin protein.

2.3 METHODS

Mice: Wild type (C57BL/10J) and *mdx* (C57BL/10ScSnDmd*mdx*/J) mice were obtained from The Jackson Laboratory (Bar Harbor, ME).

Serum preparation: Blood was collected from the hearts of mice shortly after sacrifice and centrifuged at 10,000 rpm for 15 minutes at 4°C. Supernatant was collected and stored at 4°C.

Dystrophin high-capacity adenoviral vector: The HC-Ad vector contains a full-length murine dystrophin cDNA under the control of the muscle MCK promoter, as well as the left and right viral inverted terminal repeats (ITRs) of adenovirus serotype 5 and hypoxanthine-guanine phosphoribosyl transferase (HPRT) 'stuffer' DNA. The construction of this vector has been fully described previously.[6, 10]

Mouse irradiation: Mice were irradiated at the age of 5-6 weeks in a linear accelerator (LINAC; LINAC Technologies, Orsay, France) irradiator at either a low dose (600 rads) or a high dose (900 rads). Specialized care included autoclaved cages, autoclaved food, and acidified

water (pH 2.6) from the day before irradiation until the end of each study. In the low-dose irradiated group the immune system was allowed to spontaneously reconstitute following gene transfer and in the high-dose irradiated group the mice received whole BM from wild-type C57BL/10 mice at 12 hours post-gene transfer.

Intramuscular vector injections: Irradiated and non-irradiated mice received inhalational anesthesia with isoflurane and were injected with 1.0-2.0 $x10^{10}$ genome copies of HC-AdmDYS vector intramuscularly in the TA muscle bilaterally. Each muscle was injected with a volume of 20µl in PBS, using a 28G needle (B-D; Franklin Lakes, NJ). The injections were done in an angle and the needle was pulled out slowly to make sure that most of the length of each TA muscle receives the vector.

Total muscle protein extract (TMPE) preparation: Freshly isolated mouse muscle was cut in small pieces in TEES buffer (50mM Tris-HCl pH 8.0, 5mM EGTA pH 7.4, 5mM EDTA pH 8.0, 5% SDS) and incubated on ice for 45 minutes. Samples were then sonicated briefly and centrifuged at 14,000 rpm for 20 minutes at 4°C. Supernatant was collected and stored at -80°C.

Gel electrophoresis and Western blotting: This step was done according to standard protocols. The membranes with immobilized murine dystrophin protein used for the assay were generated from wild-type C57BL/10J (B10) total muscle protein extracts (TMPE) electrophoresed on SDS-PAGE. In brief, TMPE from B10 mice were run on 5% Acrylamide gel (Bio-Rad; Hercules, CA) for 3 hours at 110V. Protein samples were transferred from the gel to nitrocellulose membrane (Amersham biosciences) for 1.5 hours at 110V at 4°C. The membrane was blocked in 5% milk/1% sheep serum/TBST (10mM Tris pH 8.0, 0.15M NaCl, 0.5mM Tween-20) overnight at 4°C. The membrane was then cut in pieces with a dystrophin protein band in each piece. A set dilution of the sera was used to examine the present or absence of an

anti-dystrophin response. The pieces of membrane were then each incubated with a diluted serum sample (1:300) in TBST for 1.5 hours, then with HRP-conjugated sheep anti-mouse IgG (GE Healthcare; Piscataway, NJ) diluted in TBST for 45 minutes at room temperature. ECL detection reagent (GE Healthcare; Piscataway, NJ) was used to detect the chemiluminescent signal and Kodak film was used for visualizing the signal. The initial exposure time was 15-30 minutes and to confirm the absence of a band in irradiated mice an additional film was exposed to the membranes overnight.

Bone marrow (BM) preparation: Tibia and femur of adult wild type mice were dissected out and their surrounding muscle was removed completely. Syringes with 26G needles were used to flush the BM out of the bones onto a cell strainer using RPMI-1460 medium supplemented with 2% fetal bovine serum (FBS), 5% penicillin/streptomycin, and 10U/ml heparin (Sigma-Aldrich; St. Louis, MO). BM was then washed with a serum-free RPMI-1460 medium (5% penicillin/streptomycin, 20mM HEPES) and counted. For adoptive transfer, cells were re-suspended in the serum-free medium at a concentration of $2.0x10^7$ cells/ml. Cell suspension (200 μl) was injected I.V. ($4\text{-}5x10^6$ cells/mouse) using a 28G needle (B-D).

Flow cytometry: Single-cell suspensions were prepared by mechanical disaggregation through a 40μm cell strainer (Fisher) into PBS. Cells were incubated with rat anti-CD4 (FITC), CD25 (APC), and Foxp3 (PE) (ebiosciences; San Diego, CA; 11-0042-82, ,17-0251-81, 12-5773-80, respectively) and rat anti-CD8 (PE), CD19 (FITC), and CD11c (FITC) (Pharmingen; San Jose, CA; 553032, 557398, and 557400, respectively) in FACS staining buffer (ebiosciences; San Diego, CA) as indicated by the manufacturer's instructions. The stained cells were then analyzed by a B-D SLR II flow cytometer.

Muscle Tissue Processing: Freshly dissected muscle was incubated in 2% paraformaldehyde/PBS on ice for 2 hours, then transferred into 30% sucrose overnight at 4°C. The next day the muscle tissue was snap-frozen in 2-methylbutane cooled with dry ice and stored at -80°C.

Immunohistochemistry of inflammatory cells: 10μm cryo-sections of muscle samples were prepared. Sections were rehydrated in PBS, blocked in peroxidase blocking reagent (DAKO Cytomation; Carpinteria, CA) for 5 minutes, and then blocked in 10% goat serum/PBS for 1 hour at room temperature. The primary antibody incubation using rat anti-CD4, Foxp3, and PD-1 (ebiosciences; San Diego, CA; 16-0041-81, 14-4771-80, and 13-9985-81, respectively) and rat anti-CD8 (Pharmingen; San Jose, CA) purified antibodies diluted in 10% goat serum/PBS were done for 1.5 hours at room temperature. Sections (except for PD-1 sections because PD-1 primary antibody was biotinylated) were incubated for 1 hour with secondary biotinylated goat anti-rat IgG (Pharmingen; San Jose, CA) diluted in DAKO antibody diluent (DAKO Cytomation; Carpinteria, CA). Sections were incubated in ABC Vectastain avidin-HRP detection solution (Vector Laboratories; Burlingame, CA) for 30 minutes at room temperature and DAB peroxidase substrate solution (Vector Laboratories; Burlingame, CA) for 4 minutes. Eosin counterstaining was performed for visualization of muscle fibers. To analyze infiltrating cells in each group, the total number of cells per cross-section of vector-injected TA muscles was counted and the average number of cells from sections from different mice was calculated.

Immuno-fluorescence detection of dystrophin and eMyoHC: 10μm cryo-sections were rehydrated with PBS, blocked first with avidin and biotin block (Vector Laboratories; Burlingame, CA), and then with mouse IgG block (Vector Laboratories; Burlingame, CA), according to the manufacturer's instructions. Incubation with primary anti-DYS or anti-eMyoHC

antibodies (Vector Laboratories; Burlingame, CA and Developmental Studies Hybridoma Bank, respectively) was done for 3 hours. Sections were then incubated with biotinylated goat anti-mouse IgG (Pharmingen; San Jose, CA) and tertiary FITC-conjugated donkey anti-goat IgG.

Statistical Analysis: In all performed studies the statistical analysis was performed by student's t-test, in which a treatment group and a control group, or two treatment groups were compared as unpaired sets. Values of variables were presented as the mean with standard deviation (SD). In all experiments, P values <0.05 were considered significant.

2.4 RESULTS

2.4.1 Low-dose irradiation delays or eliminates anti-dystrophin humoral response

I first examined the effect of a sub-lethal dose of whole-body-irradiation intended to temporarily remove the peripheral immune cells to eliminate the host peripheral immune responses against HC-Ad vector-mediated murine dystrophin expression in dystrophic muscles of adult *mdx* mice. In this study 6-week-old *mdx* mice were irradiated at 600 rads prior to gene transfer. Age-matched control groups were either not irradiated prior to dystrophin gene transfer or remained untreated for the duration of the study for each time point. Within 24 hours post-irradiation mice in both vector-injected groups each received an intramuscular injection of HC-Ad vector carrying the full-length dystrophin cDNA ($1.5\text{-}2.0 \times 10^{10}$ genome copies in each tibialis anterior (TA) muscle). Groups of treated and untreated mice were sacrificed at 4, 8, or 12 weeks post-gene delivery for analysis of blood sera for production of anti-dystrophin antibodies,

injected muscles for T cell infiltration as well as vector-mediated dystrophin expression, and draining lymph nodes for changes in B and T cell populations following gene transfer.

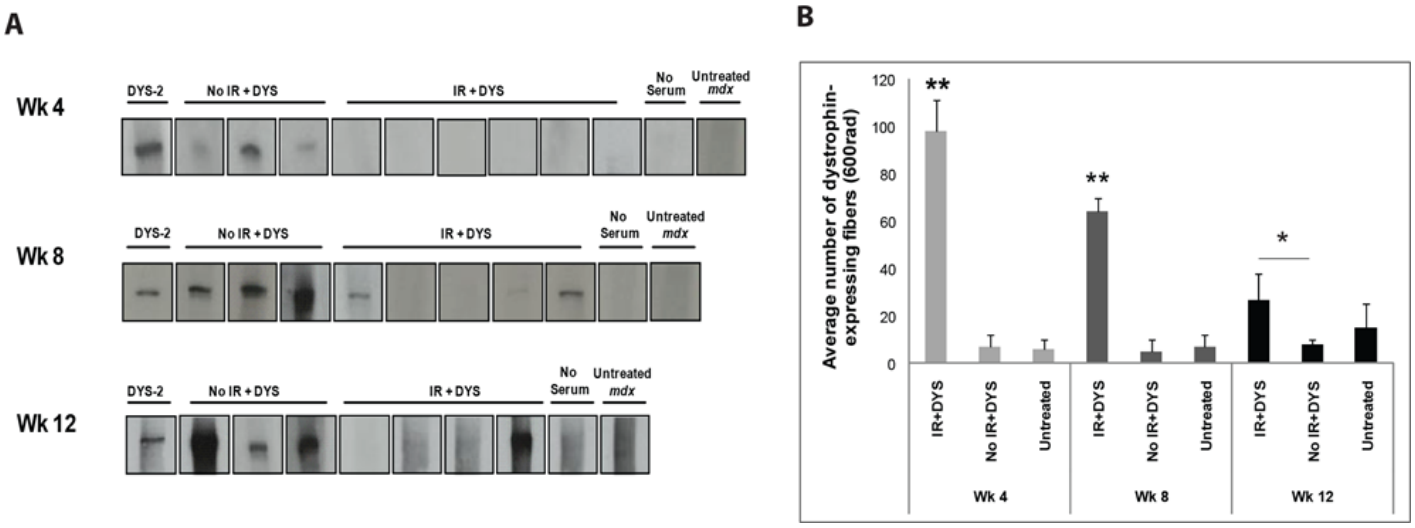

Figure 5: The anti-dystrophin humoral response and loss of muscle dystrophin expression was delayed in *mdx* mice treated with low dose irradiation followed by dystrophin gene transfer using a high-capacity adenoviral vector. (A) Sera from *mdx* mice that were irradiated at 600 rads and received an intramuscular injection of dystrophin vector (IR + DYS), received an intramuscular injection of dystrophin vector without irradiation (no IR + DYS), or received neither irradiation nor dystrophin vector (untreated *mdx*) were incubated with membrane-immobilized dystrophin protein. Sera were collected for analysis at 4, 8, and 12 weeks (Wk) after treatment (n=6 for wk 4 and n=5 for wks 8 and 12). No serum indicates a negative immunoblotting control without mouse serum primary antibody. DYS-2 indicates a positive immunoblotting control using a monoclonal anti-dystrophin antibody as the primary antibody. All experimental samples and representative control samples are presented here. (B) Dystrophin-expressing fibers per TA muscle section were counted for all experimental mice shown in (A) and untreated *mdx* control mice (n=3). Dystrophin-expressing fibers were labeled on muscle cryosections using a monoclonal anti-dystrophin antibody and were counted per section of injected TA muscles. Muscle cross-sections from different parts of each injected TA muscle were analyzed and the mean of the counted sections from treated and control mice were calculated. Dystrophin-expressing fibers were counted to be 22%, 15%, and 7% of total muscle fibers per section at weeks 4, 8, and 12, respectively. Data is expressed as mean ± standard error (SE). *(P<0.05) and **(P<0.001) indicate significant differences from untreated *mdx* control.

At 4 weeks post-gene transfer the mice that had been irradiated prior to intramuscular vector injection had no detectable anti-dystrophin humoral response (Fig. 5A, wk4). Interestingly, mice that were not irradiated prior to gene transfer all demonstrated an anti-dystrophin humoral response at as early as 4 weeks post-treatment (Fig. 5A, wk4).

At 8 weeks post-vector transfer some of the irradiated, vector-injected *mdx* mice demonstrated variable levels of anti-dystrophin humoral immunity. Three of the 5 mice

irradiated prior to gene delivery, showed a dystrophin-specific humoral response and 2 mice showed no response to dystrophin protein. All control mice that were not irradiated prior to gene transfer demonstrated a strong dystrophin-specific humoral response (Fig. 5A, wk8). In the irradiated, vector-injected group, two of the mice showed low responses and one showed a very weak response and none of them were as strong as the non-irradiated, vector-injected mice.

A third group of irradiated, vector-injected mice as well as a third group of non-irradiated, vector-injected mice were analyzed at 12 weeks post-treatment. Two of 5 irradiated, vector-injected mice produced anti-dystrophin humoral responses, while the other three did not produce anti-dystrophin antibodies (Fig. 5A, wk12). This contrasted with the control non-irradiated, vector treated mice, which all showed high levels of dystrophin-specific humoral responses (Fig. 5A and Table 1).

Table 1: Summary of humoral immune response and dystrophin expression data in low- and high-dose irradiated groups. In this table (+) indicates high level, (+/-) indicates low level, and (-) indicates an absence of response or expression. Each symbol represents an individual mouse.

		IR (600rad)+DYS	IR (900rad)+DYS+ B10 BM	IR (900rad)+ B10 BM only	No IR+DYS	Untreated
Dys+ Fibers	Wk 4	(+ + + + + ±)	(+ + + ± ±)	(- - -)	(- - -)	(- - -)
	Wk 8	(+ + + ± ±)	(+ + + ± ±)	(- - -)	(- - -)	(- - -)
	Wk 12	(+ + ± ±)	(+ ± ± ± ±)	(- - ±)	(- - -)	(- - -)
Anti-Dys Ab	Wk 4	(- - - - - -)	(- - - - -)	(- - -)	(+ + +)	(- - -)
	Wk 8	(- - - ± +)	(- - - - -)	(- - -)	(+ + +)	(- - -)
	Wk 12	(- - - +)	(+ + + + +)	(- - -)	(+ + +)	(- - -)

2.4.2 HC-Ad vector-mediated dystrophin expression in low-dose irradiated *mdx* muscles

Dystrophin expression in the vector-injected muscles of *mdx* mice was analyzed by immuno-staining of muscle cryo-sections, using an anti-dystrophin monoclonal antibody. Differentially-sized, dystrophin-expressing fibers were scattered as both individual fibers and

groups of fibers throughout the vector-injected muscle tissue of irradiated mice at 4, 8, and 12 weeks post-treatment. In contrast, vector-injected control mice that were not irradiated prior to gene transfer did not show muscle dystrophin expression above the background level of revertant fibers observed in age-matched untreated *mdx* muscle at any time-point (Fig. 5B and Table 1). The number of dystrophin-expressing muscle fibers in the irradiated, vector-treated mice decreased with time correlating temporally with the production of detectable antibodies to dystrophin protein (Fig. 5B). The reduction in the number of dystrophin-expressing fibers over time was observed even in mice that did not produce anti-dystrophin antibody, suggesting that the humoral response was not solely responsible for the decrease in dystrophin protein expression in the vector-injected muscles.

2.4.3 B cells in lymph nodes draining vector-injected muscles after low-dose irradiation

I examined peripheral immune cell reconstitution in the irradiated and non-irradiated, vector-injected mice at each time point. Since antibody production and B cell population are directly related to each other, B cell levels in the lymph nodes draining the vector-injected TA muscles were analyzed to ensure that the peripheral B cells in the irradiated mice had returned to levels comparable to untreated *mdx* mice. At all 3 time-points, the level of B cells, defined by CD19 marker, in lymph nodes draining vector-injected TA muscles of irradiated, vector-treated mice was not significantly different from untreated *mdx* mice suggesting a return to the full complement of B cells after irradiation (Fig. 6A). The number of B cells in the irradiated, vector-treated mice, however, increased gradually from week 4 to week 12. The increase in the number of B cells coincided with the development of anti-dystrophin antibodies suggesting that the self-

reconstituted B cells eventually recognized dystrophin protein as a neo-antigen and generated an anti-dystrophin immune response.

At 4 weeks post-treatment, the number of B cells in the draining lymph nodes of the non-irradiated vector-treated mice was significantly higher than both the irradiated, vector-injected and the untreated groups (Fig. 6A), suggesting an early vector-mediated B cell expansion that correlated with the high level of anti-dystrophin humoral response observed in vector-treated mice. These findings, therefore, indicated that the peripheral immune cells were fully back and functional following the whole-body irradiation.

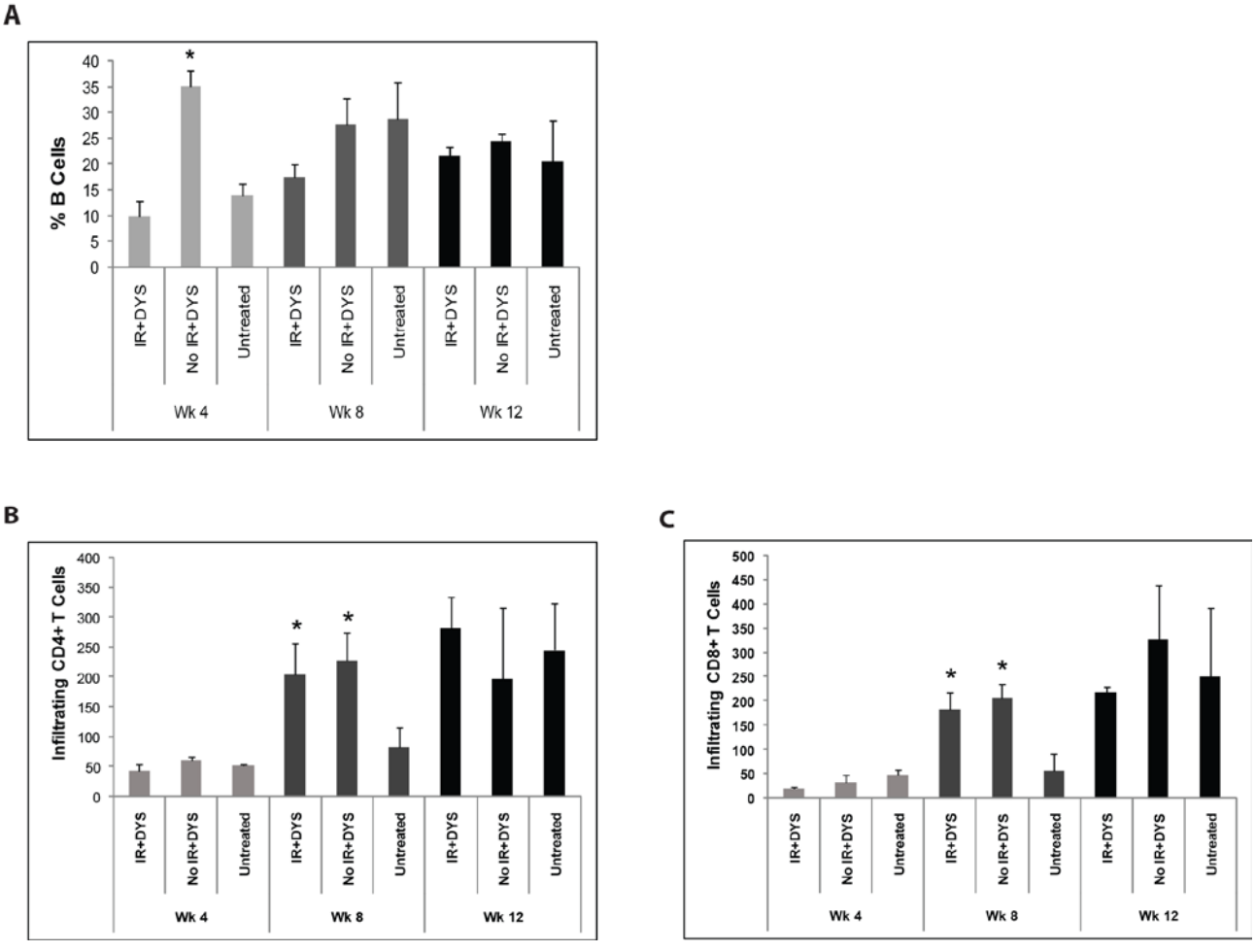

Figure 6: Infiltrating T cells, but not peripheral lymphocytes from vector-injected muscle draining lymph nodes in low-dose irradiated *mdx* mice are different from untreated *mdx* muscle. (A) The B cell

population in draining lymph nodes was analyzed by flow cytometry in low-dose irradiated and non-irradiated *mdx* mice at 4, 8, and 12 weeks post-treatment. Infiltrating $CD4^+$ (B) and $CD8^+$ (C) T cells were examined by immunohistochemistry in the treated muscles of low-dose irradiated and non-irradiated *mdx* mice at the three time points mentioned above. Number of mice in each group is the same as in Fig. 1. Data is expressed as mean ± standard error (SE). * ($P<0.05$) indicates significant difference from untreated *mdx* control.

2.4.4 T-cell infiltrates in vector-injected muscles after low-dose irradiation

I also investigated the infiltration of $CD4^+$ and $CD8^+$ T cells in vector-injected muscles of *mdx* mice to assess the cellular immune reaction following recombinant murine dystrophin gene expression in the treated muscles. Since muscle infiltration of $CD4^+$ and $CD8^+$ T cells is a disease-associated phenotype of *mdx* mouse muscle and is thought to promote muscle fiber necrosis, infiltrating T cells are seen in dystrophic *mdx* muscles with and without dystrophin gene transfer. Therefore, I compared the number of infiltrating T cells in vector-injected muscle, with or without irradiation, to age-matched untreated muscle. This allowed me to both assess the return of T cells to the peripheral tissues following whole-body irradiation, and investigate the level of infiltrating T cells following vector-mediated dystrophin expression.

Levels of infiltrating $CD4^+$ and $CD8^+$ T cells in muscles of low-dose irradiated, vector-treated or non-vector-treated mice were not significantly different from untreated mice at week 4 post-treatment (Fig. 6B and 6C). At week 8 the levels of both $CD4^+$ and $CD8^+$ T cells increased in the vector-injected TA muscles of both irradiated and non-irradiated hosts compared to the untreated muscle, indicating perhaps the development of a T cell-mediated host immune response following dystrophin vector expression at this time point that was not affected by irradiation (Fig. 6B and 6C). By week 12, levels of $CD4^+$ and $CD8^+$ T cells had increased in all groups to similar levels (Fig. 6B and 6C). Since T cell levels were similar in the low-dose irradiated and non-irradiated groups following vector-mediated dystrophin expression, I decided

to look at the pattern of T cell infiltration in the muscles of these mice to see if there is a difference between the two treated muscles.

Interestingly, the pattern of T cell infiltration for both $CD4^+$ and $CD8^+$ T cells observed on muscle sections was consistently different in the vector-treated, irradiated mice compared to the vector-treated, non-irradiated group (Fig. 7). At week 4, T cells were scattered throughout the muscle tissue in irradiated vector-injected *mdx* muscles, similar to those in age-matched untreated *mdx* muscles. In the non-irradiated mice that received the dystrophin vector, however,

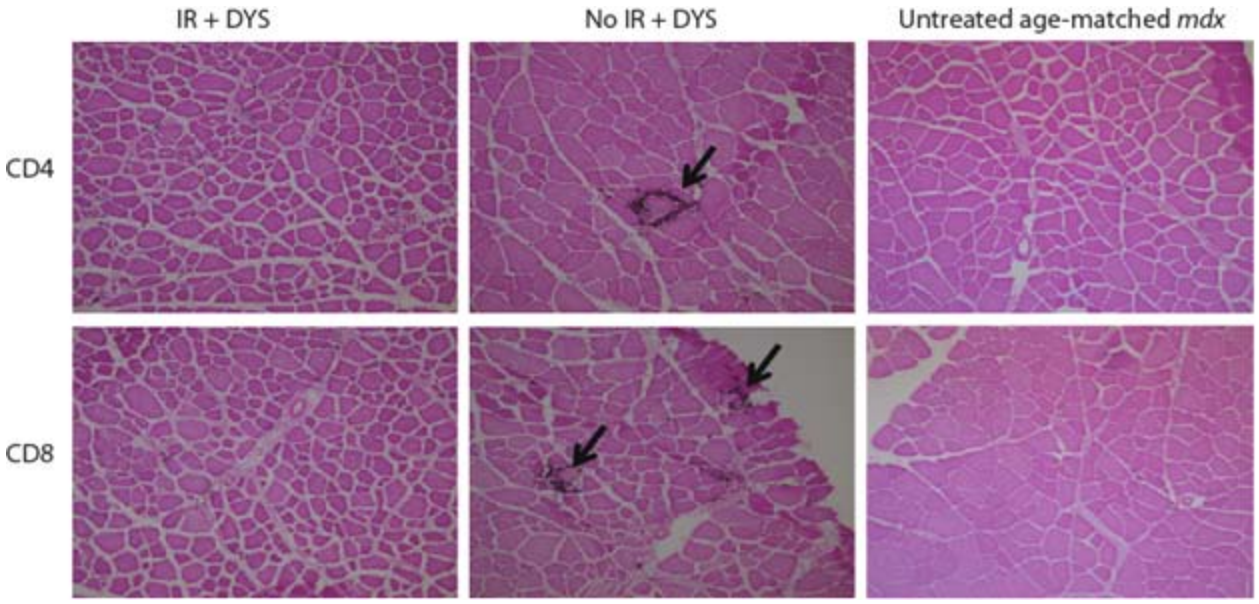

Figure 7: Pattern of T cell infiltration in low-dose irradiated, vector-injected muscle is significantly different from non-irradiated, vector-injected muscle. Infiltrating CD4+ and CD8+ T cells were examined by immunohistochemistry in treated and control muscle at week 4, 8, and 12 post-treatment. This figure shows a representative image of week 8 analysis. Arrows indicate single or multiple muscle fibers surrounded with infiltrating T cells.

in addition to scattered infiltrating cells, large numbers of cells were seen to cluster around one or multiple adjacent fibers, and groups of necrotic muscle fibers were seen as early as 4 weeks post-treatment. This observation, plus the fact that dystrophin-expressing fibers were absent in non-irradiated, vector-injected muscles as early as 4 weeks post-vector injection suggested that

perhaps the dystrophin-expressing fibers were targeted by a specific cell-mediated host immune response in the absence of irradiation prior to gene transfer. In the irradiated group a similar pattern was only seen in the vector-injected muscles at week 12 post-treatment (data not shown).

2.4.5 High-dose irradiation delays anti-dystrophin humoral response

I next examined the effect of removing central and peripheral immune cells of *mdx* mice by 900 rads whole-body irradiation prior to intramuscular dystrophin gene delivery ($1.5x10^{10}$ genome copies in each TA muscle). The immune systems of irradiated mice were then

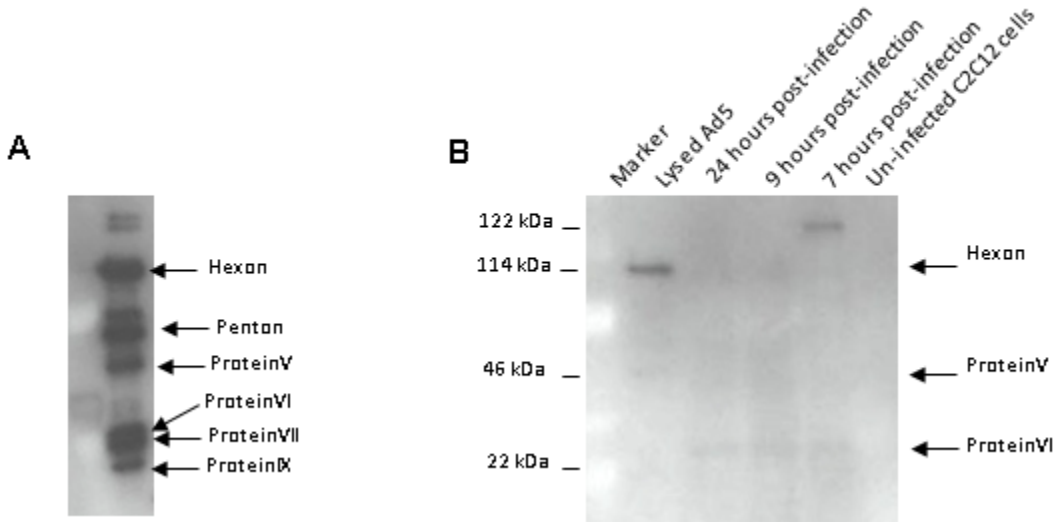

Figure 8: Temporal analysis of degradation of adenoviral capsid proteins following infection of muscle cells. C_2C_{12} myoblast cells were infected with adenoviral vector and incubated in myoblast differentiation media (DMEM supplemented with 10% FBS and 1% Penicillin and Streptomycin) at 37°C for 48 hours. Samples of cells were harvested at different time points to examine viral protein degradation by comparing an intact viral vector sample (A) to cell lysis from C_2C_{12} cell cultures (B). At 24 hours only a faint band in position of proteinVI of the viral capsid protein was observed and all other proteins had been degraded.

reconstituted by transfer of either wild-type BM from adult B10 donors or *mdx* BM. Similar to the low-dose irradiation studies, mice were analyzed at 4, 8, or 12 weeks post-treatment. I had three control groups: one that underwent only gene delivery without receiving other treatments,

one that was irradiated and received adoptive transfer of only B10 BM without gene transfer, and one that was left untreated for each time point (Table 1).

I considered what would be an expected time course to clear the initial load of vector capsid proteins. To explore this experimentally, C_2C_{12} myoblast cells were infected *in vitro* with Ad vector. Harvested cell samples at various time points for 48 hours were assayed for Ad vector capsid proteins by Western blot. Nearly all Ad capsid proteins were degraded *in vitro* by 24 hours post-infection (Fig. 8A and 8B). Despite likely differences between viral protein degradation *in vitro* and *in vivo*, the *in vitro* results gave me an approximate time point for progressing to the *in vivo* studies. Balancing the desire to maximize the time for viral proteins to degrade prior to BM transfer with a need to minimize mouse loss due to high dose irradiation, I performed BM transfer at about 15 hours post-gene transfer. BM transfer consisted of tail vein intravascular (IV) injections of 4.0-5.0 x 10^6 BM cells.

At 4 weeks post-gene transfer none of the mice that had been irradiated and received either B10 or *mdx* BM produced anti-dystrophin antibodies. However, the vector-injected mice that had not been irradiated prior to gene transfer produced anti-dystrophin antibodies at this time point, similar to the control group in the low-dose irradiation study (Fig. 9A). Unlike the low-dose study group, however, in which a few mice produced anti-dystrophin antibodies at week 8 post-treatment, at this time-point none of the irradiated vector-injected mice that received B10 BM produced an anti-dystrophin humoral response (Fig. 9A). Nonetheless, in the group of mice that was irradiated and received *mdx* BM prior to dystrophin vector transfer, 3 out of 5 mice produced anti-dystrophin humoral response at this time point (Fig. 9A). By 12 weeks post-treatment I observed anti-dystrophin antibody production in all of the irradiated, vector-injected mice that received adoptive transfer of B10 or *mdx* BM (Fig. 9A). Therefore, a high-dose

irradiation followed by B10 BM transfer seemed to be the most successful study group in delaying the anti-dystrophin host immune response for the longest period of time. Nonetheless, the group with *mdx* BM still had advantages over the non-irradiated, vector-injected group because it only partially showed anti-dystrophin humoral response at 8 weeks post-gene transfer, compared to the non-irradiated vector-injected group that showed a full anti-dystrophin response at as early as 4 weeks post-gene transfer.

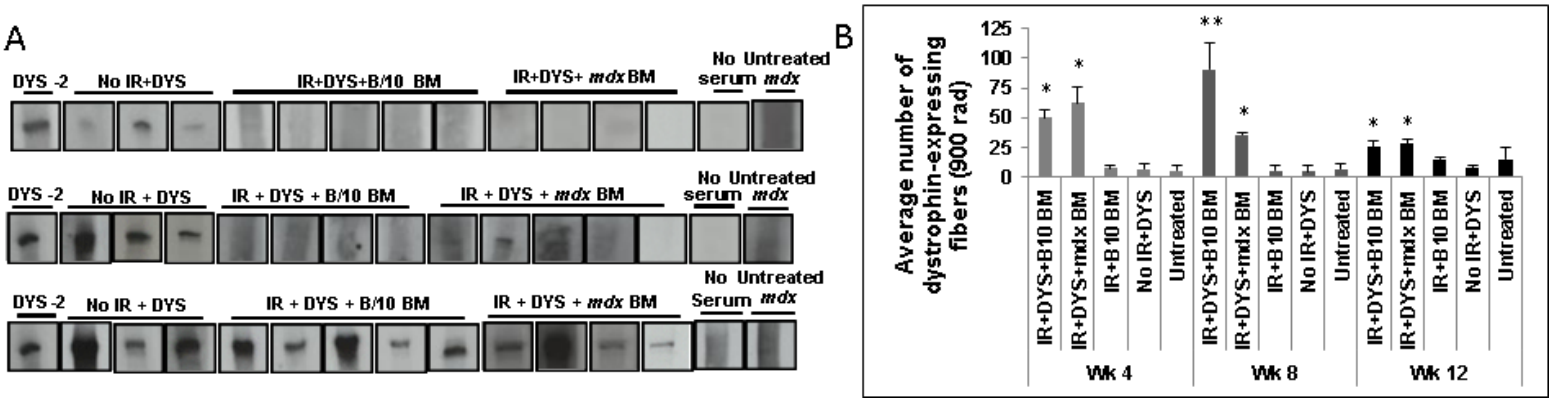

Figure 9: The anti-dystrophin humoral response and loss of muscle dystrophin expression was delayed in *mdx* mice treated with high dose irradiation followed by dystrophin gene transfer using a high-capacity adenoviral vector. (A) Sera from *mdx* mice that 1) were irradiated at 900 rads and received an intramuscular injection of dystrophin vector (IR + DYS+B10 BM), 2) were irradiated and received B10 BM only (IR+B10 BM), 3) received an intramuscular injection of dystrophin vector without irradiation (no IR + DYS), or 4) received neither irradiation nor dystrophin vector (untreated *mdx*) were incubated with membrane-immobilized dystrophin protein. Sera were collected from treated and control mice at 4, 8, and 12 weeks (Wk) after treatment (n=5 for IR+DYS+B10 BM and n=3 for all control groups). No serum indicates a negative control without mouse serum labeling. DYS-2 indicates a positive immunoblotting control using a monoclonal anti-dystrophin antibody as the primary antibody. All experimental samples and representative control samples are presented here. (B) Dystrophin-expressing fibers per TA muscle section were counted for all experimental mice shown in (A) and all untreated *mdx* control mice. Dystrophin-expressing fibers were labeled on muscle cryosections using an anti-dystrophin monoclonal antibody and were counted per cryosection of treated TA muscles. Muscle cross-sections of each vector-injected and uninjected TA muscles were analyzed and the mean of the fibers counted in sections from treated and control mice were calculated. Dystrophin-expressing fibers were counted to be 10%, 20%, and 5% of total muscle fibers per section at weeks 4, 8, and 12, respectively. Data is expressed as mean ± standard error (SE). *(P<0.05) and **(P<0.001) indicate significant differences from untreated *mdx* control.

2.4.6 HC-Ad vector-mediated dystrophin expression in high-dose irradiated *mdx* muscles

Dystrophin protein expression in the muscles of treated *mdx* mice was assessed by immunohistological assay, as before. In the high-dose irradiation group I first determined that adoptive transfer of B10 BM alone does not give rise to dystrophin-expressing fibers above the level in untreated age-matched *mdx* muscles, for at least 12 weeks post-treatment (Fig. 9B).

At all time-points post-treatment, dystrophin expression was significantly higher in the muscles of the irradiated mice that had received vector and either B10 or *mdx* BM compared to the control groups (Fig. 9B, wk4). At week 4 post-vector injection, the number of dystrophin-expressing fibers was similar between the two irradiated, vector-injected groups, regardless of the source of BM reconstitution. At week 8 post-treatment, however, the level of dystrophin expression increased significantly in B10 BM recipient mice that received dystrophin vector, but decreased in *mdx* BM recipient mice that received dystrophin vector (Fig. 9B; wk8). Therefore, at week 8 post-treatment the level of dystrophin expression in B10 BM recipient mice was significantly higher than all other treated or untreated groups at that time point. The subsequent decline in dystrophin expression level at week 12 post-treatment in both BM recipient groups correlated with the production of anti-dystrophin antibodies in all the irradiated, BM and vector-treated mice.

2.4.7 B cells in the host lymph nodes draining the vector-injected muscles after high-dose irradiation and BM transfer

The lymph nodes draining vector-treated muscles of the high-dose irradiated mice were analyzed to examine peripheral lymphocyte levels. The percentage of B cells in the draining

lymph nodes was similar among all groups at 4 and 8 weeks post-treatment (Fig. 10A). By week 12 post-treatment the B cell levels in all groups had decreased (Fig. 10A, wk12). This observation mainly indicated that following high-dose irradiation and BM transfer the host immune system had successfully been reconstituted and that the donor cells (both B10- and *mdx*-derived cells) were in the peripheries at high levels.

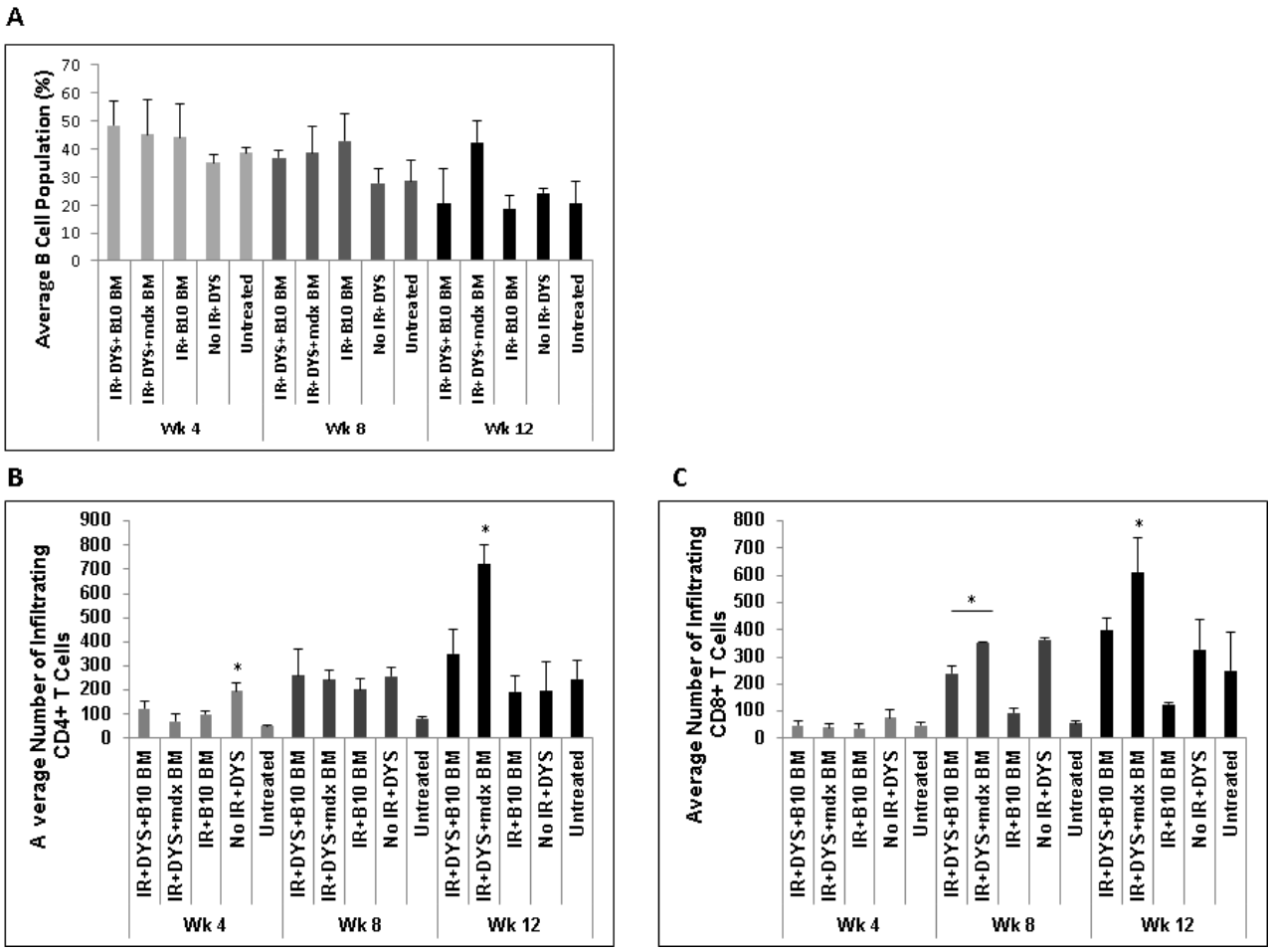

Figure 10: Infiltrating T cells and peripheral lymphocytes from vector-injected, muscle-draining lymph nodes in high dose irradiated *mdx* mice differ from untreated *mdx* mice. (A) The B cell population in draining lymph nodes was analyzed by flow cytometry in high-dose irradiated and non-irradiated *mdx* mice at 4, 8, and 12 weeks post-treatment. Infiltrating $CD4^+$ (B) and $CD8^+$ (C) T cells were examined by immunohistochemistry in the treated muscles of high-dose irradiated and non-irradiated *mdx* mice at the three time points mentioned above. Number of mice in each group is the same as in Fig. 4. Data is expressed as mean ± standard error (SE). * (P<0.05) indicates significant difference from untreated *mdx* control.

2.4.8 T-cell infiltrates in vector-injected muscles after high-dose irradiation and BM transfer

I also looked at T cell infiltration in the treated muscles at the same time points. At week 4 post-treatment, $CD4^+$ T cells were significantly lower in the muscles of the irradiated mice that had received dystrophin vector and B10 or *mdx* BM compared to the non-irradiated, vector-injected mice, but $CD8^+$ T cells were at comparable levels (Fig. 10B and 10C). Starting at week 8, however, muscle infiltrating $CD4^+$ and $CD8^+$ T cells were different in the irradiated, vector-injected mice that received B10 BM compared to those that had received *mdx* BM. In the B10 BM recipient mice the level of $CD8^+$ T cells was significantly lower than the control groups, where $CD8^+$ T cells in the *mdx* BM recipient group had increased to a level even higher than the non-irradiated, vector-injected mice (Fig. 10B and 10C). The levels of infiltrating $CD4^+$ T cells did not change significantly at week 12 post-treatment compared to week 8 in the irradiated, vector-injected group that received B10 BM, but increased significantly in the *mdx* BM recipient group to a level that was even significantly higher than the non-irradiated, vector-injected control group (Fig. 10B and 10C).

Infiltrating T cells were observed scattered throughout in the high-dose irradiated, vector-injected muscles of the B10 BM recipient group at all time points in contrast to clustering around specific muscle fibers in non-irradiated, vector-injected muscle. The observed difference in the pattern of muscle T cell infiltration between irradiated, vector-injected, B10 recipients and the non-irradiated vector-injected control group in the high-dose irradiation study was similar to that observed between irradiated, vector-injected group and non-irradiated, vector-injected control group in the low-dose irradiation study. In the irradiated, vector-injected group that received *mdx* BM, however, a significant difference was observed compared to the irradiated, vector-injected,

B10 BM recipients; large numbers of infiltrating T cells had surrounded groups of muscle fibers (Fig. 11). This difference was especially noticeable at week 12 post-treatment. This result clearly showed a difference between the two sources of BM that were used to reconstitute the high-dose-irradiated mice, at a cellular level.

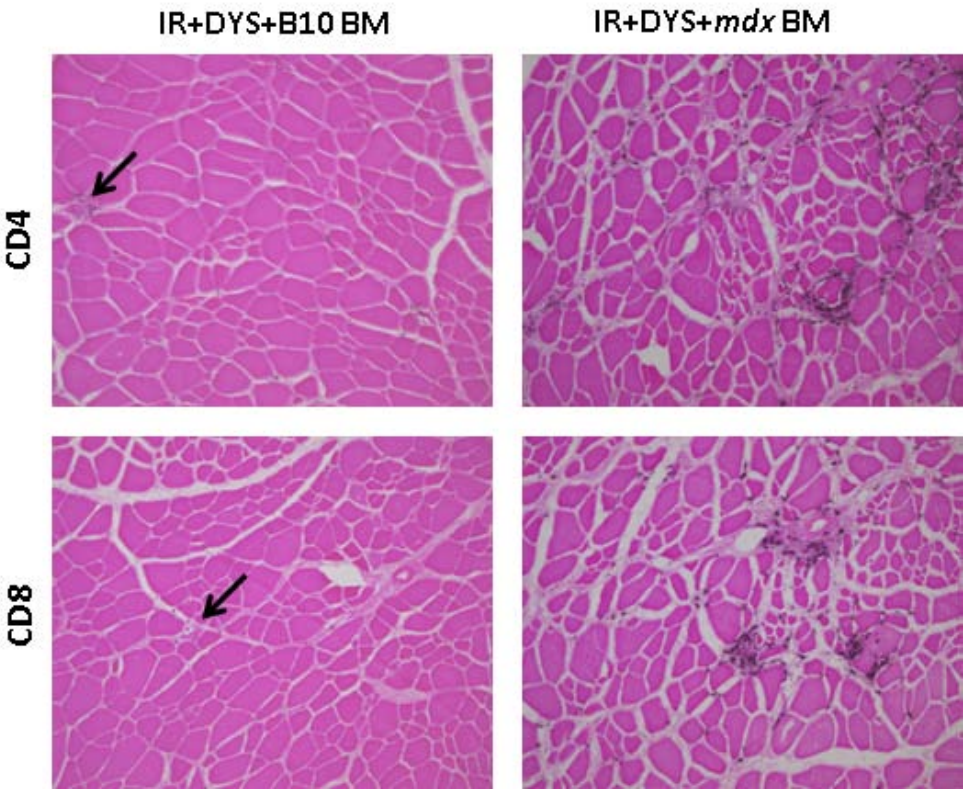

Figure 11: T cell infiltration pattern difference between B10 BM recipients and *mdx* BM recipients. Infiltrating $CD4^+$ and $CD8^+$ T cells were examined by immunohistochemistry in high-dose irradiated, vector-injected BM recipients and *mdx* recipients at week 4, 8, and 12 post-treatment. This figure shows a significant difference between the two BM recipients from week 8 analysis. Arrows show small areas with groups of T cells in irradiated, vector-injected, B10 BM recipients.

2.4.9 Regulatory T cells in treated muscles

An important cell population in modulating immunity is the Foxp3-expressing regulatory T cell subset.[82, 83] I compared the infiltration of Treg cells in treated muscles at each time point to explore a possible role of these cells in modulating the immunity that I observed in treated *mdx* mice. In the low-dose irradiation study, the number of muscle-infiltrating Treg cells was comparable at each time point for the vector-treated groups with and without irradiation (Fig.

12A). In this treatment groups there was a decrease from week 4 to week 8 followed by an increase from week 8 to week 12, a pattern that was also observed in untreated *mdx* mouse muscle, suggesting a contribution of the dystrophic disease progression in muscle. In the high-dose irradiation study group, however, there was a significant increase in Treg cell infiltration starting at week 8 post-treatment in the irradiated, vector-injected mice, that received either B10 or *mdx* BM, but not in the non-irradiated, vector-injected mice (Fig. 12B). The number of Treg cells further increased at week 12 post-treatment and remained significantly higher in the irradiated, vector-injected B10 BM recipients as compared to the non-irradiated, vector-injected mice (Fig. 12B). At this time point Tregs increased in *mdx* BM recipients as well, but this increase was not significant compared to their level at week 8. This observation again indicated dissimilarity between B10 and *mdx* BM recipients in terms of host immune system following gene transfer.

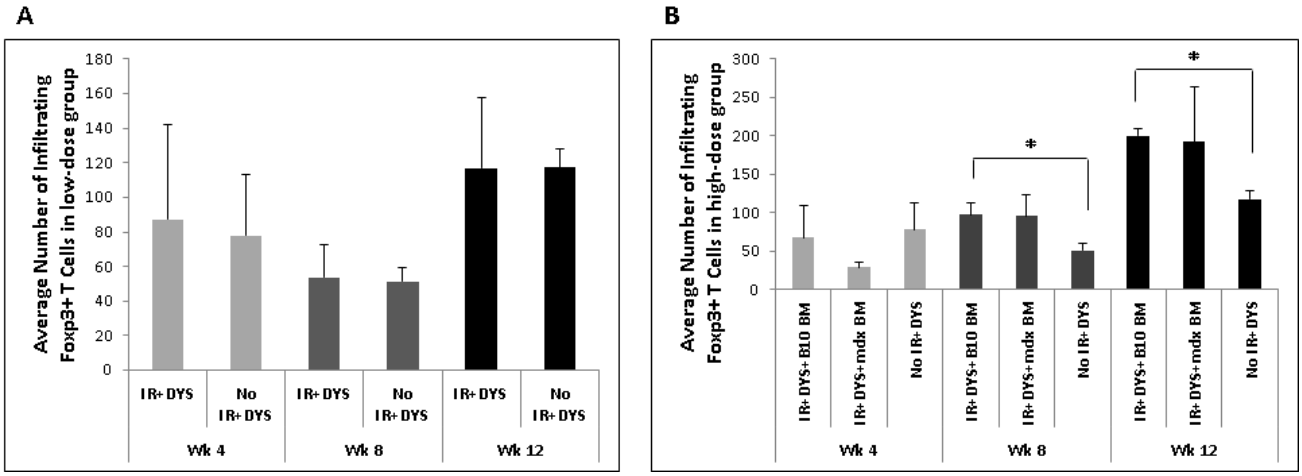

Figure 12: Regulatory T cell infiltration in treated *mdx* muscles. Infiltrating Foxp3$^+$ regulatory T cells in treated muscles of both low-dose (A) and high-dose (B) irradiated and control groups were analyzed by immunohistochemistry at 4, 8, and 12 weeks post-treatment. Number of mice in each group is the same as in Fig. 1 and Fig.4. Data is expressed as mean ± standard error (SE). * (P<0.05) indicates significant difference from untreated *mdx* control.

I also examined the level of muscle infiltration of programmed death-1 (PD-1) expressing cells that have been shown to have immune regulatory effects.[125] There was no significant difference in the number of PD-1$^+$ muscle-infiltrating cells in different treatment groups at different time points in mice receiving either low-dose or high-dose irradiation and either B10 or *mdx* BM (data not shown).

2.5 DISCUSSION

In studies on therapeutic gene replacement for DMD, the immune response of the dystrophic host to vector-mediated dystrophin protein expression is an important limiting factor with the potential to cause rejection of recombinant dystrophin. Further understanding of the immunological reactions against dystrophin, immune mechanisms that may modulate anti-dystrophin immunity and manipulations that may reduce anti-dystrophin immune responses in a dystrophic host provides the potential to develop approaches designed to prolong dystrophin expression in the recipient of therapeutic gene transfer.

A temporal analysis of the effect of irradiation alone or irradiation combined with BM transfer on the immunity against vector-mediated full-length dystrophin expression in *mdx* muscle has not been previously reported. Previous studies have examined the effects on dystrophic muscle of adoptive transfer of wild-type BM to irradiated *mdx* mice,[126] muscle precursor cells to irradiated *mdx* mice,[127] or BM-derived stem cells to non-irradiated *mdx* mice.[128] In the current study I limited the dose of irradiation to a maximum of 900 rads, as in other studies,[127] to avoid damage to muscle stem cells.[129] The use of the HC-Ad vector for gene delivery allowed exposure to all epitopes of an expressed full-length murine dystrophin cDNA.

In these studies, BM transfer alone to *mdx* mice did not increase the level of muscle dystrophin expression over the background level in untreated *mdx* mice at any time-point, which confirmed the findings of others.[126, 130] Therefore, the dystrophin expression observed after dystrophin vector delivery following irradiation and BM transfer is vector-mediated.

Our findings demonstrate that irradiation of the dystrophic *mdx* host prior to intramuscular vector injection led to a delayed, reduced, or absent humoral response against dystrophin protein. The changes in anti-dystrophin immunity in treated mice allowed me to study the progression of host immunity against dystrophin and its effect on dystrophin expression over a period of 12 weeks. The observation that both low-dose and high-dose irradiation delayed the development of an anti-dystrophin antibody response is significant compared to the previous findings of more rapid host immunity against dystrophin.[9]

The delay in development of an anti-dystrophin humoral response was greater in the high-dose irradiated group compared to the low-dose irradiated group, as no anti-dystrophin humoral response was observed at 8 weeks post-treatment. These results suggest that the more complete immune cell depletion (both central and peripheral immune cells) and reconstitution with dystrophin-tolerant immune cells results in greater suppression of anti-dystrophin immunity induced by dystrophin gene transfer to the dystrophin-deficient *mdx* host.

The level of $CD4^+$ and $CD8^+$ T cell infiltration in untreated dystrophic *mdx* muscle[17, 18] and in dystrophin HC-Ad vector-injected *mdx* muscle[7, 10] has been previously investigated. It has been shown that the dystrophic pathology of *mdx* mouse muscle includes high levels of $CD4^+$ and $CD8^+$ T cell infiltration in the damaged tissue that is thought to promote the pathology associated with the disease.[17, 18] If the immune system in a dystrophic host is not down-regulated, the high level of T cell infiltration in the damaged tissue increases following vector injection, due

to a natural response of the immune system against the vector and transgene particles. It has been shown that specific immune modulations that lead to absent or reduced T cells in the *mdx* mouse can ameliorate the disease process in muscle[16, 17] and, in a gene transfer setting, reduce immunity against dystrophin protein in the host.[6, 10] The present study compared the levels of infiltrating $CD4^+$ and $CD8^+$ T cells in vector-injected muscles to untreated *mdx* mouse muscle at different time points. Thus, I evaluated the role that these T cell sub-populations may play in a gene therapy setting as both disease and the immunity against recombinant dystrophin progressed. In contrast to the observed correlation with the anti-dystrophin humoral response, irradiation prior to gene transfer does not seem to affect levels of infiltrating T cells in vector-injected muscles of the *mdx* host during 12 weeks post-treatment, especially in the low-dose irradiation group. Interestingly, however, the pattern of T cell infiltration in muscle tissue did appear to be affected by irradiation.

In the early stages after gene transfer, the finding of muscle fibers surrounded and invaded by T cells was only observed in the non-irradiated, vector-injected muscles. This focal pattern of T cell infiltration was not observed in the irradiated vector-injected muscles of the low-dose irradiation study group, the high-dose irradiated, vector-injected, B10 recipients, or in untreated *mdx* mice. Nonetheless, a similar pattern of T cell infiltration to the non-irradiated, vector-injected group was observed in the irradiated-vector-injected, *mdx* BM recipients, and in fact it seemed to be more extreme in the *mdx* BM recipients at wk 12 post-treatment. Double-staining of T cells and dystrophin in the attacked fibers was not feasible because only necrotic fiber remnants remained. Nonetheless, the distinctive pattern of T cell infiltration plus the absence of dystrophin-expressing fibers in non-irradiated, vector-injected muscles at early stages post-gene transfer suggested that transduced fibers were targeted for immune attack. The same

pattern of T cell infiltration has also been observed in previous studies in which transfer of muscle precursor cells from dystrophin-normal donors to irradiated *mdx* mice that had received mixed BM from muscle cell-donors and *mdx* donors, resulted in infiltration of $CD4^+$ and $CD8^+$ T cells around dystrophin-expressing muscle fibers at 3-4 months post-treatment.[127] In addition, in a recent clinical trial, in which AAV vector-mediated expression of a mini-dystrophin protein was analyzed in DMD patients, dystrophin-specific T cell responses were observed in some of the patients.[131] This T cell response was shown to be dystrophin-specific and correlated with a loss of dystrophin protein expression in these patients,[131] supporting the possibility of a T cell-mediated rejection of vector-mediated dystrophin expression in the treated *mdx* mice in my studies.

In addition to the control mice that were treated with the vector without irradiation, I also observed a rapid decrease in vector-mediated dystrophin expression in irradiated, vector-injected, *mdx* BM recipients at week 8 following post-treatment, but did not see the same result in the irradiated, vector-injected B10 BM recipients. Therefore the presented data indicated that this pattern of T cell infiltration, along with the humoral response that was seen in the non-irradiated, vector-injected mice and the *mdx* BM recipient mice precluded successful vector-mediated gene expression in the *mdx* muscle.

Overall, my analysis of T cell infiltration in treated *mdx* muscle indicates that even though $CD4^+$ and $CD8^+$ T cells infiltrates are found in irradiated, vector-treated *mdx* muscles, this host immune response does not lead to an immediate rejection of dystrophin protein in the vector-injected muscles of irradiated mice for at least 12 weeks post-treatment. In contrast, T cell infiltration in the vector-injected muscles of non-irradiated mice appeared to reject dystrophin-expressing fibers, suggested by the pattern of T cell infiltration surrounding muscle fibers and

the absence of dystrophin expression as early as 4 weeks post-treatment. This pattern of T cell infiltration also correlated with production of anti-dystrophin antibody production in these mice. One limitation of the analysis of cellular immunity observed in treated or untreated *mdx* muscle was that the antigens inducing immunity cannot be specifically determined.

To decipher the mechanism underlying the delayed and diminished host immune response observed in both low- and high-dose irradiated groups, I examined infiltration of $Foxp3^+$ Treg cells and PD-1^+ T cells in treated and control *mdx* muscles. Only Treg cells were different among the groups. I have previously observed infiltrating $Foxp3^+$ Treg cells to be at slightly higher levels in untreated *mdx* mouse muscles compared to age-matched B10 muscles (unpublished data). In this study, I observed even higher levels of Treg cells in vector-injected muscle of *mdx* mice 8 and 12 weeks after receiving high-dose irradiation and reconstitution of the immune system, especially with B10 BM. This increase in Treg cells suggested their role in the diminished anti-dystrophin immune response observed in the setting of central and peripheral immune cell depletion followed by reconstitution. In contrast, Treg cells did not appear to play a role in the delayed immunity associated with depletion of the peripheral immune cells alone followed by self-reconstitution (low-dose irradiation). Taken together, these results suggest a role for Treg cells in the suppression of immunity induced by dystrophin vector gene transfer followed by reconstitution of the central immune system. The adoptive transfer of whole BM from B10 mice may have contributed to the relatively high level of Treg cells in treated muscles since the transferred BM is likely to contain cells tolerant to B10 self-antigens, including muscle proteins. The exact role of Treg cells in the setting of dystrophin gene delivery to dystrophic muscle will require further study.

In summary, I show that manipulation of the host immune system by irradiation of the adult dystrophic *mdx* mouse prior to intramuscular HC-Ad dystrophin vector delivery results in a delayed and diminished humoral immune response to vector-mediated dystrophin protein expression in the adult *mdx* mouse. The delay in the response correlated with a significantly slower rate of elimination of vector-mediated dystrophin protein expression in treated *mdx* muscle compared to muscle of non-irradiated vector-injected mice. Although there are technical limitations to determining the antigen specificity of T cells infiltrating muscle, the pattern of T cells surrounding and invading muscle fibers suggests that viral vector-mediated dystrophin transduction contributes to the antigenic targets, particularly since this pattern of T cell infiltration is diminished and delayed by irradiation. The failure to eliminate anti-dystrophin humoral and T cell-mediated immunity with both central and peripheral immune depletion achieved by irradiation supports the contention that dystrophin expression in dystrophin-deficient, dystrophic muscle is immune-stimulatory, a finding which has significant ramifications for dystrophin gene replacement therapy.

3.0 ANALYSIS OF EFFECTS OF RAPAMYCIN (RAPA) TREATMENT ON DYSTROPHIC MUSCLES OF *MDX* MOUSE SKELETAL MUSCLES

3.1 RATIONALE

The following study was conducted to test the hypothesis that treatment of dystrophic muscles of the *mdx* mouse with the immunosuppressive drug, RAPA, will ameliorate the dystrophic pathology of *mdx* mouse muscles due to its effects on down-regulating the *mdx* mouse immune system.

3.2 INTRODUCTION

The ongoing necrosis of dystrophic skeletal muscle leads to a substantial infiltration in the diseased muscle of immune cells, that may be, in part, autoreactive. Previous studies of the depletion of $CD4^+$ and $CD8^+$ T cells in the *mdx* mouse resulted in a significantly lower level of muscle fiber necrosis and fibrosis in dystrophic muscle.[16, 17] Furthermore, the only proven treatment for human DMD, prednisone, is a known immunosuppressant,[132] suggesting that down-regulation of the immune system in a dystrophic setting may have therapeutic effects.

RAPA, also referred to as sirolimus, has been widely used for immune suppression in the setting of allograft organ or tissue transplantation.[133-135] Compared to other immunosuppressant

drugs such as cyclosporine A, studies have shown that RAPA has lower toxicity and leads to a greater decrease in T cell immunity.[136] One of the mechanisms of the selective effect of RAPA is an increase in Foxp3^{+} Treg cell survival and function, which has been shown *in vitro* and *in vivo*.[111, 112] Therefore, I hypothesized that RAPA treatment of the *mdx* mouse would diminish dystrophic pathology of the diseased muscle tissue.

In mammals RAPA binds to the mammalian target of rapamycin (mTOR), which is a highly conserved serine/threonine protein kinase that is expressed in all cell types[137] and regulates cell growth and protein synthesis.[138-140] There are two distinct mTOR complexes known as complexes 1 and 2 (mTORC1 and mTORC2, respectively), that vary in both structure and function, and respond differentially to nutrients, cellular energy, and growth factors, as reviewed by Zhou and Huang.[141] The relative importance of mTORC1 and mTORC2 varies among different tissues. In skeletal muscle, mTORC1, which is RAPA-sensitive, has been shown to play an important role in muscle growth.[142, 143] Skeletal muscle mTOR activation levels have been followed longitudinally in the *mdx* mouse.[144] However, comparison of mTOR activation between age-matched *mdx* and wild-type B10 mice have not been reported. Findings of these studies are important for a better molecular understanding of DMD pathology and can lead to potential therapeutic approaches for this disease.

Because RAPA is a potent immunosuppressant and has the potential to modulate muscle growth and regeneration, I studied its effect on the pathology of dystrophic muscle. I examined the effects of RAPA on *mdx* muscle pathology and mTOR activation when administered either locally or systemically during the peak of *mdx* disease-related muscle degeneration and regeneration. Given that systemic immune suppression may be associated with adverse outcomes, I used two strategies to treat *mdx* mice with RAPA: 1) local treatment of *mdx* TA

muscles by injection of RAPA-containing microparticles, and 2) systemic treatment of *mdx* mice through RAPA-containing water. The systemic treatment in this study allowed for the analysis of the Dia muscle, which is not easily accessible by direct intramuscular injection.

3.3 METHODS

Mice: Wild type (C57BL/10J) and *mdx* (C57BL/10ScSnDmd*mdx*/J) mice were obtained from The Jackson Laboratory (Bar Harbor, ME).

Cell Culture: Splenocytes were isolated from adult C57BL/10J mice by passing spleen tissue through a cell strainer (Fisher) to obtain a single-cell suspension. $CD4^+CD25^+$ cells were then isolated from the total splenocytes using Miltenyi Biotech separation columns, according to the manufacturer's instructions. In brief, cells were first incubated with an antibody cocktail and passed through a negative separation column to remove all cells other than $CD4^+$ cells from the splenocytes. The removed cells were irradiated at 3000 rads to be used as antigen presenting cells (APCs) in cell cultures. Next, the $CD4^+$ cells were labeled with an anti-CD25 antibody and passed through a positive separation column to separate $CD25^+$ cells from other $CD4^+$ cells, to obtain $CD4^+CD25^+$ cells. These separated cells were then incubated in DMEM medium supplemented with 10% FBS, 1% non-essential amino acids, 2mM L-glutamine, 50µM 2-beta mercapto-ethanol (2-ME) and 10% peniciline and streptomycine, in standard conditions (37°C and 5% CO_2). Rapamycin was added to the cells at 48 hours post-culture and was added again every 4-5 days for a total of 14 days. Cells were harvested after 2 weeks in culture and were analyzed by flow cytometry (BD SLR II flow cytometer) for percentage of $CD4^+CD25^{hi}Foxp3^+$ cells.

Total muscle protein extract (TMPE) preparation: Freshly isolated mouse muscle was cut into small pieces in TEES buffer (50mM Tris-HCl pH 8.0, 5mM EGTA pH 7.4, 5mM EDTA pH 8.0, 5% SDS) and incubated on ice for 45 minutes. Samples were then sonicated briefly and centrifuged at 14,000 rpm for 20 minutes at 4°C. Supernatant was collected and stored at -80°C.

Rapamycin-containing PLGA microparticle preparation: RAPA-releasing microparticles were produced as previously described.[145] In brief, RAPA microparticles were produced using the single emulsion-evaporation technique that involved forming an emulsion of an organic solution containing rapa and PLGA in a bulk aqueous solution, through high speed homogenization (3000 rpm). Following evaporation of the organic solvent (dichloromethane), microparticles formed in the aqueous solution were freeze-dried for subsequent use in experiments.

RAPA/H_2O preparation and administration: To prepare the *mdx* mouse drinking water containing RAPA (LC Laboratories; Woburn, MA), 1.5mg/kg mouse of RAPA (about 0.04mg/day/mouse) was dissolved in autoclaved water. Fresh RAPA/H_2O was prepared every 7-10 days. The amount of RAPA added to the *mdx* mouse drinking water was calculated to approximately match the amount of RAPA released from the RAPA-containing microparticles that were used in the local treatment group on a daily basis.

Intramuscular microparticle injection: Six-week-old *mdx* mice were injected intramuscularly with rapamycin-containing microparticles (RAPA beads) in sterile PBS in the TA muscle (30µl per TA muscle). Control mice either received empty microparticles (blank beads) or were left untreated. Microparticles were injected into the TA muscles with a high-dose injection (1mg RAPA per injection) initially, followed by half-dose injection (0.5 mg RAPA per

injection) 2 weeks later. Mice were collected 6 weeks after initiation of treatment, thus allowing for complete degradation of PLGA microparticles by the time of analysis.

Gel electrophoresis and Western blotting: Gel electrophoresis and Western blotting were done according to standard protocols. Briefly, TMPE from B10 mice was electrophoresed on 5% Acrylamide gel (Bio-Rad; Hercules, CA) for 3 hours at 110V. Protein samples were transferred from the gel to nitrocellulose membrane (Amersham biosciences) for 1.5 hours at 110V at 4°C. The membrane was blocked in 5% milk/1% sheep serum/TBST (10mM Tris pH 8.0, 0.15M NaCl, 0.5mM Tween-20) overnight at 4°C. The membrane containing TMPE was incubated with primary rabbit anti-mouse total mTOR and phosphorylated mTOR antibodies (Cell Signaling Technology; Danvers, MA) in 5%BSA/TBST for 1.5 hours, then with HRP-conjugated donkey anti-rabbit IgG (GE Healthcare; Piscataway, NJ) diluted in TBST for 45 minutes at room temperature. ECL detection reagent (GE Healthcare; Piscataway, NJ) was used to detect the chemiluminescent signal and Kodak film was used for visualizing the signal. The initial exposure time was 5-15 minutes. To confirm the absence of a band, an additional film was exposed to each membrane overnight. The bands were then analyzed using MCID image analysis software (Interfocus Imaging Ltd.; Cambridge, England) to find density of each band. Values for phosphor-mTOR were then divided by total mTOR values for evaluation of the level of mTOR activation for each mouse.

Flow cytometry: Single-cell suspensions of cultured cells were prepared by mechanical disaggregation through a 40μm cell strainer (Fisher) into PBS. Cells were incubated with rat anti-CD4 (FITC), CD25 (APC), and Foxp3 (PE) (ebiosciences; San Diego, CA; 11-0042-82, ,17-0251-81, 12-5773-80, respectively) and rat anti-CD8 (PE), CD19 (FITC), and CD11c (FITC) (Pharmingen; San Jose, CA; 553032, 557398, and 557400, respectively) in FACS staining buffer

(ebiosciences; San Diego, CA) as indicated by the manufacturer's instructions. The stained cells were then analyzed by a BD SLR II flow cytometer.

Muscle Tissue Processing: Freshly dissected muscle was incubated in 2% paraformaldehyde/PBS on ice for 2 hours, then transferred into 30% sucrose overnight at 4°C. The next day the muscle tissue was snap-frozen in 2-methylbutane cooled with dry ice and stored at -80°C.

Immunohistochemistry of inflammatory cells: 10μm cryo-sections of muscle samples were prepared. Sections were rehydrated in PBS, blocked in peroxidase blocking reagent (DAKO Cytomation; Carpinteria, CA) for 5 minutes, and then blocked in 10% goat serum/PBS for 1 hour at room temperature. The primary antibody incubation using rat anti-CD4 and Foxp3 (ebiosciences; San Diego, CA) and rat anti-CD8 (Pharmingen; San Jose, CA) purified antibodies diluted in 10% goat serum/PBS was performed for 1.5 hours at room temperature. Sections were incubated for 1 hour with secondary biotinylated goat anti-rat IgG (Pharmingen; San Jose, CA) diluted in DAKO antibody diluent (DAKO Cytomation; Carpinteria, CA). Sections were incubated in ABC Vectastain avidin-HRP detection solution (Vector Laboratories; Burlingame, CA) for 30 minutes at room temperature and DAB peroxidase substrate solution (Vector Laboratories; Burlingame, CA) for 4 minutes. Eosin counterstaining was performed for visualization of muscle fibers. To analyze infiltrating cells in each group, the total number of cells per cross-section of vector-injected TA muscles was counted and the average number of cells from sections from different mice was calculated.

Immunofluoresence staining for necrotic fibers: Muscle fiber necrosis was evaluated through fluorescent-conjugated IgG staining of muscle cryo-sections. In brief, muscle cryo-sections (10μm thick) were re-hydrated with PBS, then blocked with 1% gelatin/1% rabbit

serum/PBS for 15 minutes. Samples were next washed with PBS-G wash buffer (0.2% gelatin/PBS) and incubated with Alexa-488-conjugated rabbit anti-mouse IgG (Invitrogen; Carslbad, CA) for 1 hour, following with PBS-G washes.

Immunofluorescence staining for regenerating fibers: Muscle fiber regeneration was evaluated through staining for embryonic myosing heavy chain (eMyoHC) staining. 10μm cryo-sections were rehydrated with PBS, blocked first with avidin and biotin block (Vector Laboratories; Burlingame, CA), and then with mouse IgG block (Vector Laboratories; Burlingame, CA), according to the manufacturer's instructions. Samples were also briefly blocked with 10% goat serum/PBS for 15 minutes at room temperature. Incubation with primary anti-eMyoHC antibodies (Developmental Studies Hybridoma Bank) was done for 3 hours at room temperature. Sections were then incubated with biotinylated goat anti-mouse IgG (Pharmingen; San Jose, CA) and tertiary FITC-conjugated donkey anti-goat IgG.

Statistical Analysis: In all performed studies the statistical analysis was performed by student's t-test, in which a treatment group and a control group, or two treatment groups were compared as unpaired sets. Values of variables were presented as the mean with standard deviation (SD). In all experiments, P values <0.05 were considered significant.

3.4 RESULTS

3.4.1 RAPA induces Treg proliferation in vitro in the presence of APCs

Before examining the effects of RAPA on cellular infiltrates and pathology of dystrophic muscles in *mdx* mice, I investigated the effects of RAPA on murine T cells *in vitro*. Therefore, I

isolated T cells from adult wild-type mice to incubate in the presence of RAPA with or without APCs. Spleen-derived B10 $CD4^+CD25^+$ T cells, separated through cell separation columns (Miltenyi biotech), were plated at a density of 250,000 cells per plate in a 6-well plate. Irradiated APCs (3000rads) were also added at a 1:1 ratio to the plates. Forty-eight hours after plating the cells, RAPA was added and the cells were incubated for 14 days. Fresh RAPA was added every 48 hours. A significant increase in the number of $Foxp3^+$ $CD4^+$ cells was observed in response to RAPA and APCs (Fig. 13). This observed increase in Foxp3+ Treg cells in the presence of RAPA was in agreement with the literature on using RAPA for Treg expansion.

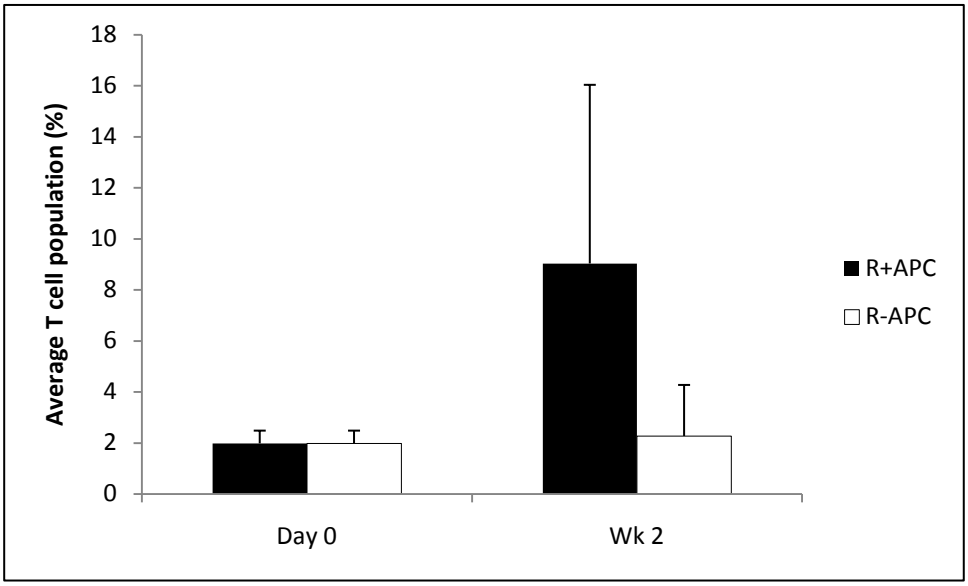

Figure 13: *In vitro* treatment of murine CD4+CD25+ cells with RAPA. Splenocytes isolated from wild-type C57BL/10 mice were enriched for $CD4^+CD25^+$ T cells through column separation techniques and were incubated with irradiated antigen presenting cells and treated with RAPA for 2 weeks. Harvested cells were analyzed by flow cytometry to evaluate the percentage of $CD4^+CD25^+Foxp3^+$ T cells following the treatment. (R) indicates RAPA and (APC) indicates antigen presenting cells. The blue bar shows the increase in percentage of $CD4^+CD25^+Foxp3^+$ Tregs in the presence of APCs and RAPA.

3.4.2 Short-term comparison between injecting *mdx* muscles with RAPA microparticles (slow release) and RAPA solution (fast release)

Following the *in vitro* analysis of the effects of RAPA on Treg cells, I performed a short-term study on whether to study the effects of RAPA on *mdx* muscle in vivo through injection of RAPA-containing microparticles or RAPA solution. This study was designed for confirming the benefits of a slow-release system for RAPA in vivo before beginning the long-term study using microparticles. For this study, TA muscles of age-matched *mdx* mice were injected with either

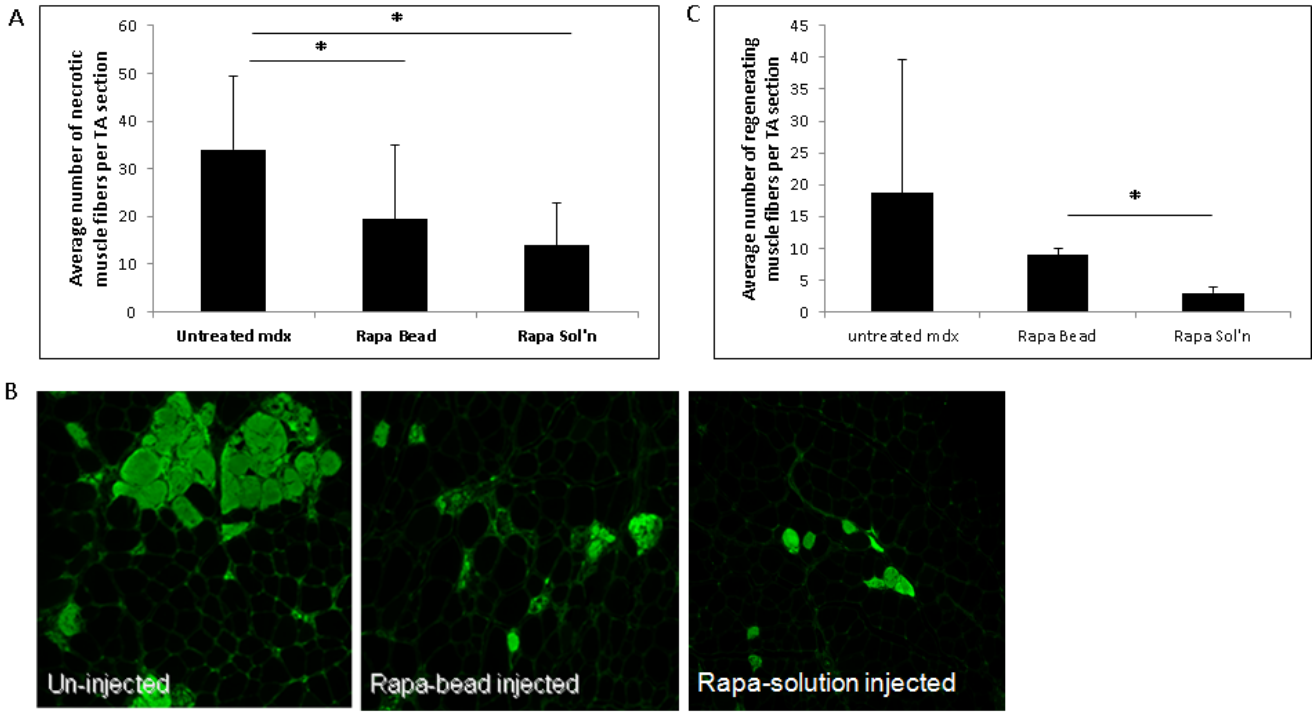

Figure 14: Muscle fiber necrosis and regeneration following short-term RAPA treatment of *mdx* muscles. Two weeks following RAPA treatment, injected TA muscles of *mdx* mice and age-matched untreated *mdx* control mice were collected for analysis. IgG staining was used to examine the level of necrotic fibers (A and B) and embryonic myosin heavy chain (eMyoHC) staining was used to evaluate the level of muscle fiber regeneration (C) in muscle tissues. * indicates p value <0.05 and ** indicates p value <0.01.

RAPA microparticles or RAPA solution. Through both injections about 1mg of RAPA was injected into each TA muscle and the volume of injection in both groups was 30µl per TA

muscle. Mice were sacrificed and TA muscles were collected for analysis at 2 weeks post-treatment. In my analysis I evaluated the levels of muscle fiber necrosis, muscle fiber regeneration, and Foxp3^{+} cell infiltration between the two treatments and compared them to untreated age-matched *mdx* TA muscles. I observed that both RAPA microparticles and RAPA solution had lead to a significantly lower level of muscle fiber necrosis compared to age-matched untreated *mdx* muscle (Fig. 14A and 14B). The level of muscle fiber necrosis in both RAPA-treated groups was comparable with the level of RAPA solution group only slightly lower.

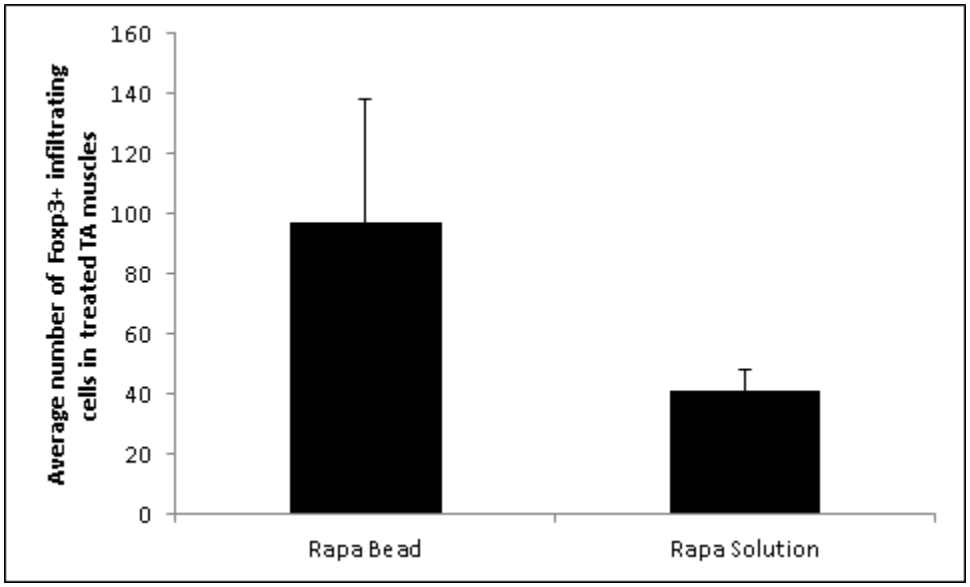

Figure 15: Foxp3+ T cell infiltration following short-term RAPA treatment of *mdx* muscle tissue. Two weeks following RAPA treatment, infiltration of Foxp3+ T cells was analyzed through immunohistochemistry in the injected TA muscles of RAPA-treated *mdx* mice.

The decrease in muscle fiber necrosis, naturally lead to a significantly lower level of muscle fiber regeneration in both RAPA-treated groups (Fig. 14C). However, the level of muscle fiber regeneration in the RAPA-solution-injected muscle was significantly lower than that in the RAPA-microparticle-injected muscles, suggesting a more severe effect of the drug on muscle fiber growth and protein synthesis when RAPA is injected into the muscles at once. Comparing the level of infiltrating Foxp3^{+} cells in the RAPA-treated muscle tissue indicated a beneficial

effect of a slow-release of RAPA over a fast release, as $Foxp3^+$ cells were significantly higher in the RAPA-microparticle-injected muscles compared to age-matched untreated *mdx* muscles, but the level of $Foxp3^+$ cells was comparable to the untreated mice in the RAPA solution-injected muscles (Fig. 15). Therefore, I decided to use RAPA-microparticles for a local treatment strategy in *mdx* muscles for the rest of my studies. This decision was based on the findings of the more detrimental effects of injection of RAPA solution on muscle fiber regeneration and $Foxp3^+$ cell survival in the treated muscle tissue.

3.4.3 RAPA lowers infiltrating effector T cells, but not $Foxp3^+$ regulatory T cells in *mdx* muscles

Given that infiltration of effector T cells in dystrophic muscles of *mdx* mice plays an important role in the pathology associated with the disease, I hypothesized that RAPA would improve the pathology of *mdx* muscle by reducing the level of infiltrating T cells. Both local and systemic treatments began at 6 weeks of age and were continued for 6 weeks prior to sacrifice and analysis. TA and Dia muscles were analyzed in the systemic RAPA-treated group, but only TA muscles were analyzed in the local RAPA-treated group, because transfer of microparticles would not allow for a systemic RAPA delivery.

To assess the immunosuppressant effects of RAPA on dystrophic muscle tissue, the levels of infiltrating $CD4^+$ and $CD8^+$ T cells in TA muscles of *mdx* mice were assessed. There was a significant decrease in total infiltrating $CD4^+$ T cells in *mdx* muscles treated with local or systemic RAPA compared to untreated or blank bead-injected *mdx* muscles (Fig. 16A). A significant decrease in $CD8^+$ T cells was also observed in *mdx* TA muscles with systemic

RAPA-treatment, but not with local treatment (Fig. 16A). Infiltrating $CD4^+$ and $CD8^+$ T cells were observed at highest levels in areas of necrosis in untreated *mdx* muscle tissue (Fig. 16B). In *mdx* muscles treated with RAPA, however, infiltration of both $CD4^+$ and $CD8^+$ T cells was scattered in the tissue (Fig. 16B). In contrast to the effects on $CD8^+$ and total $CD4^+$ T cell levels, $Foxp3^+$ T cells were not significantly reduced in *mdx* TA muscles treated with local or systemic RAPA compared to the untreated or blank-bead-treated age-matched muscles (Fig. 16A). This observation supported the literature in other tissues on the effects of RAPA on survival of $Foxp3^+$ Tregs.[111, 112, 146]

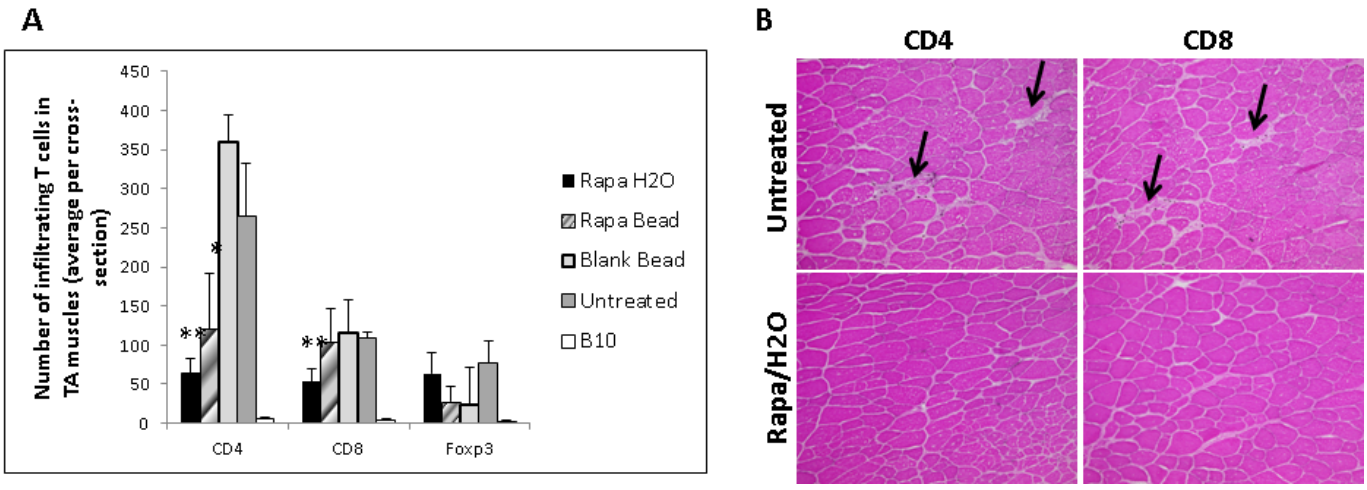

Figure 16: T cell infiltration in RAPA-treated *mdx* muscles. Six weeks following either local (Rapa Bead) or systemic (Rapa H2O) RAPA treatment of 6-week-old *mdx* mice, TA muscles were collected for analysis. T cell infiltration was evaluated by immunohistochemistry on muscle cross-sections of TA muscles. T cell numbers are presented as average number of cells per cross-section (A). Arrows in panel (B) show areas of muscle fiber necrosis with $CD4^+$ or $CD8^+$ T cell infiltrates. * indicates p value < 0.05 and ** indicates p value < 0.01.

3.4.4 RAPA lowers muscle fiber necrosis and regeneration in *mdx* muscles

Infiltrating T cells contribute to the pathologic process of muscle fiber necrosis in

dystrophic muscles of the *mdx* mouse and DMD patients.[17, 18] Therefore, I quantitatively examined the level of muscle fiber necrosis in RAPA-treated and age-matched untreated *mdx* muscles (Fig. 17).

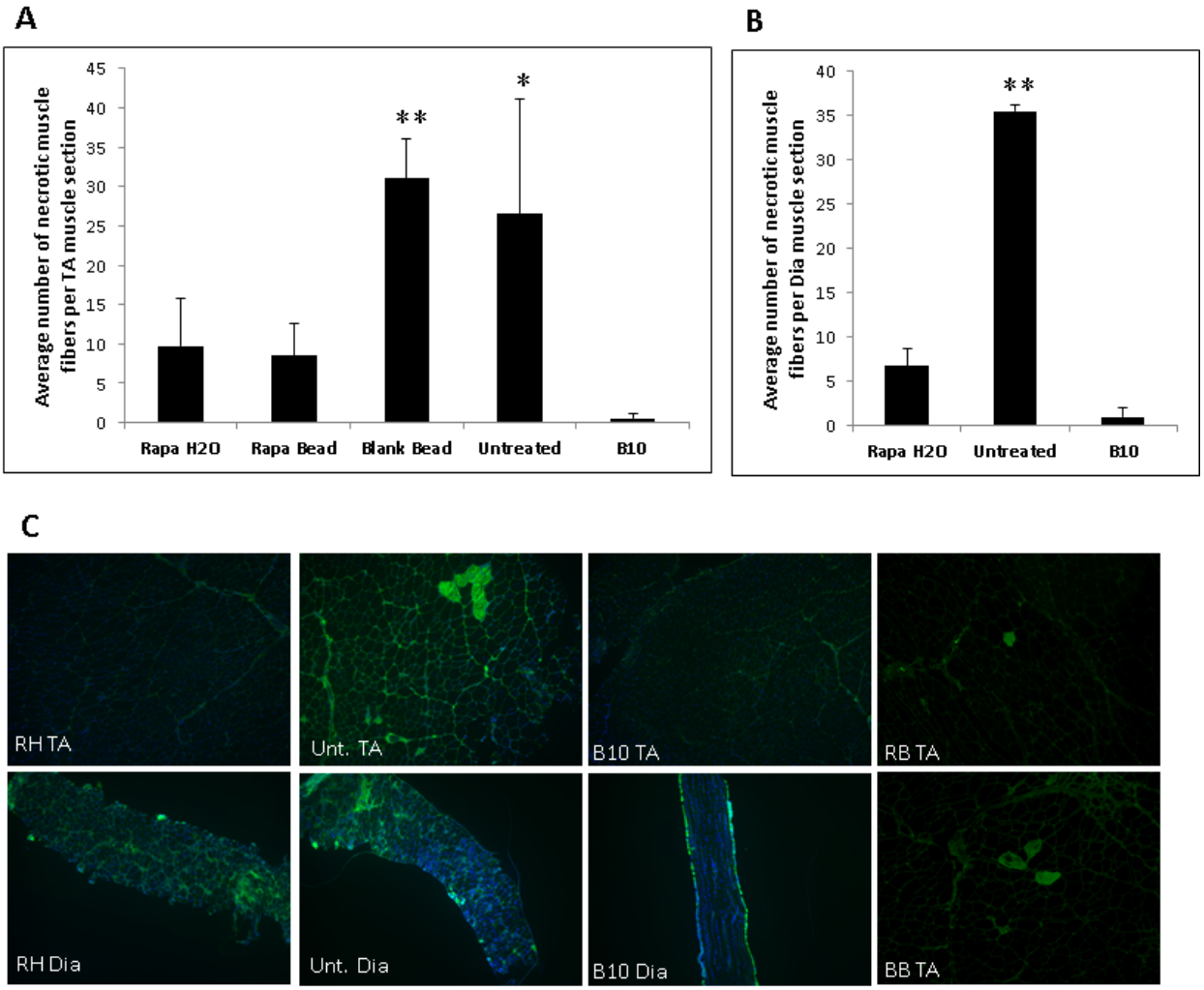

Figure 17: RAPA treatment lowers muscle fiber necrosis in *mdx* muscles. TA muscles of all RAPA treated mice and Dia muscles of systemic RAPA-treated *mdx* mice only, were collected for analysis six weeks following RAPA treatment. Both local and systemic RAPA treatments in lead to significant decreases in muscle fiber necrosis in both TA (A) and Dia (B) muscle tissues. IgG staining using a FITC-conjugated anti-mouse IgG was used to label necrotic muscle fibers (C). Graphs show average number of necrotic muscle fibers per TA or Dia muscle cross-sections. * indicates p value < 0.05 and ** indicates p value < 0.01.

The total number of necrotic fibers per TA muscle cross-section, as determined by incubation with fluorescently labeled IgG, in mice with systemic or local RAPA treatment was

significantly less compared to age-matched untreated *mdx* muscle (Fig.17A and 17C). Furthermore, with systemic RAPA treatment, I also observed significantly decreased necrosis in the diaphragm (Fig. 17B and 17C).

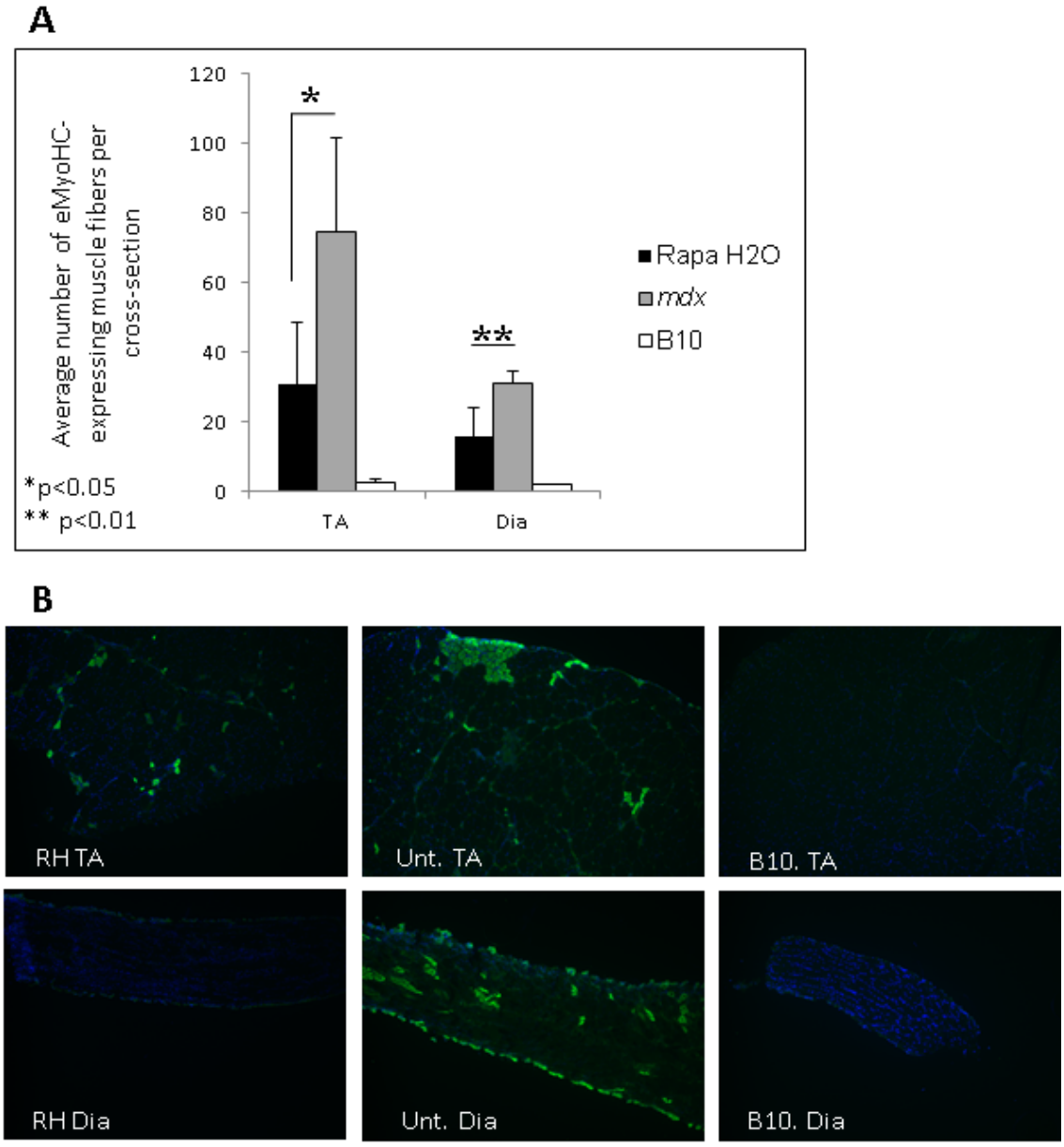

Figure 18: Muscle fiber regeneration in RAPA-treated *mdx* muscles. TA muscles of all RAPA treated mice and Dia muscles of systemic RAPA-treated *mdx* mice only, were collected for analysis six weeks following RAPA treatment. Both local and systemic RAPA treatments lead to significant decreases in muscle fiber regeneration in both TA and Dia muscles (A). Data from systemic RAPA-treatment study is shown as a representative. eMyoHC staining was used to evaluate muscle regeneration (B). Graph in panel (A) shows average number of newly regenerated muscle fibers per TA or Dia muscle cross-sections. * indicates p value < 0.05 and ** indicates p value < 0.01.

The necrotic muscle fibers in untreated *mdx* mice were observed in large groups of fibers in muscle tissue, whereas in the RAPA-treated mice the fewer necrotic fibers were observed as scattered small groups or individual fibers within the muscle tissue (Fig. 17C).

I also observed a significantly lower level of muscle fiber regeneration, as indicated by embryonic myosin heavy chain (eMyoHC) expression in TA and Dia muscle fibers of systemic RAPA-treated and TA muscle of local RAPA-treated mice compared to age-matched untreated or blank bead-treated *mdx* muscles (Fig. 18A and 18B). Figure 20 shows representative data from the systemic RAPA-treated group.

3.4.5 mTOR activation differs between TA and Dia muscles of *mdx* mouse

The activation of mTOR by phosphorylation in the presence of energy, nutrients, or growth factors is known to be important for muscle fiber growth[147] and RAPA can block this activation. Therefore, I examined the level of mTOR activation in TA and Dia muscles of 6-week-old (during active necrosis) and 12-week-old (after the peak of active necrosis) age-matched untreated *mdx* and wild-type B10 mice by Western blot. Surprisingly, mTOR activation was not significantly different between TA muscle of untreated *mdx* and age-matched B10 mice at 6 and 12 weeks of age (Fig. 19A and 19B). In both *mdx* and B10 TA muscles the level of mTOR activation decreased between 6 and 12 weeks of age.

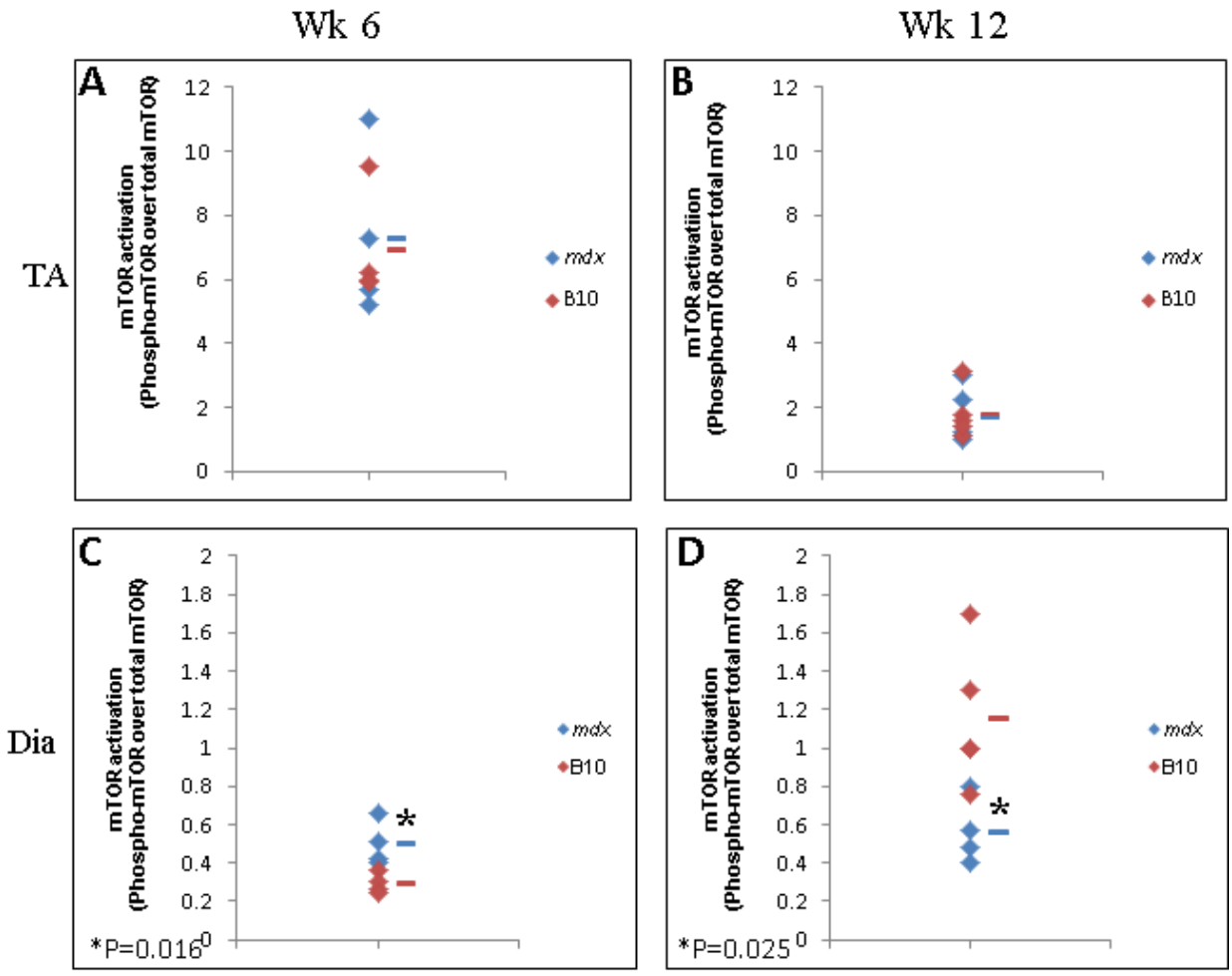

Figure 19: mTOR phosphorylation in 6- and 12-week-old *mdx* versus B10 mice. mTOR activation (phosphorylated mTOR over total mTOR) was analyzed in TA and Dia muscles of 6- and 12-week-old *mdx* mice and was compared to age-matched B10 mice. Western blot analysis was used to measure mTOR phosphorylation in total muscle protein extracts from muscle samples. (A-D) show average blot intensity of the labeling for each tissue type and (E) is a representative image of the blots analyzed. * indicates p value < 0.05.

However, mTOR activation was significantly different between *mdx* and B10 mice in Dia muscle at both 6 and 12 weeks of age (Fig. 19C and D). At 6 weeks of age, mTOR activation was lower in B10 Dia muscle than in *mdx* Dia muscle. I observed that mTOR activation in Dia muscles of B10 mice increased from 6 to 12 weeks of age. However, mTOR activation remained at similar levels in *mdx* Dia muscles over the same timeframe.

3.4.6 mTOR activation is affected in Dia but not TA muscles of RAPA-treated mice

RAPA directly interacts with mTOR in skeletal muscles. Therefore, I compared activation of mTOR in skeletal muscles of *mdx* mice that received systemic and local RAPA treatment with age-matched untreated or blank bead-treated *mdx* mice. RAPA treatment led to a significantly lower level of mTOR activation in Dia muscle compared to untreated age-matched *mdx* Dia muscle (Fig. 20A). However, surprisingly, neither local nor systemic RAPA treatment resulted in a significant change in mTOR activation in TA muscles of treated *mdx* mice compared to untreated age-matched *mdx* mice (Fig. 20B and 20C).

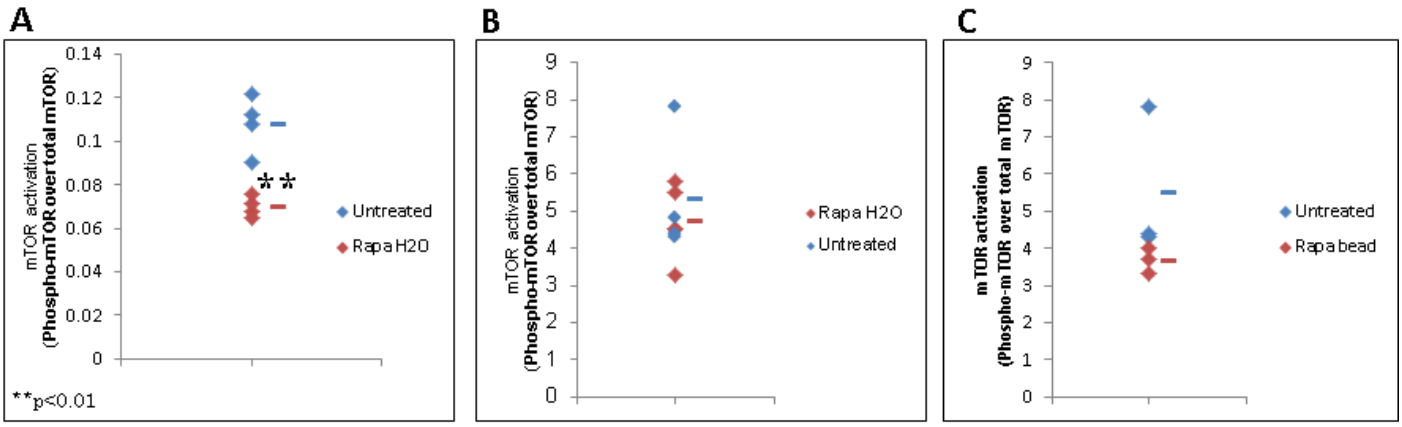

Figure 20: mTOR phosphorylation following RAPA treatment. mTOR activation (phosphorylated mTOR over total mTOR) was assessed in Dia and TA muscles of systemically RAPA-treated (A and B) and only TA muscles of locally RAPA-treated (C) *mdx* mice at 6 weeks following treatment. This analysis was done by Western blot analysis and the graphs show average blot intensity for each tissue type. ** indicates p value < 0.01.

3.5 DISCUSSION

In the study presented here I explored the effect of RAPA on inflammation, necrosis, regeneration, and mTOR activation in the dystrophic *mdx* mouse muscles. The effects of

different immunosuppressant drugs, including cyclosporine A, prednisone, and deflazacort, have been investigated in dystrophin-deficient muscles, as reviewed by Iannitti *et.al*.[148] The study presented here, however, is the first report of RAPA treatment for dystrophin-deficient skeletal muscle of the *mdx* murine model for DMD, a model in which a genetic defect in dystrophin expression promotes inflammatory cell infiltration in muscle tissue. Others have previously shown that administration of RAPA in organ transplantation led to a higher rate of graft survival.[149-151]

RAPA has been widely used in various studies to expand and/or select for $Foxp3^+$ Tregs.[111, 112, 146, 152, 153] In contrast to the inhibitory effect of RAPA on proliferation of effector T cells, Tregs proliferate and function in the presence of RAPA.[111, 112, 146, 147] I show here that $Foxp3^+$ cells survive in dystrophic skeletal muscle when RAPA is administered for 6 weeks to *mdx* mice, whereas total $CD4^+$ and $CD8^+$ T cells, decrease significantly. The most prominent effect was observed with systemic RAPA treatment. The selective proliferation of Foxp3+ Treg cells coupled with a decrease in effector T cells could lead to improvements in morphology and function of dystrophic skeletal muscle.

This study demonstrates that RAPA administration ameliorates the dystrophic phenotype of *mdx* muscle. Based on prior data showing a beneficial effect of RAPA for organ transplantation and the knowledge that modulation of immunity ameliorates dystrophic changes in muscle in muscular dystrophy, I hypothesized that RAPA would decrease necrosis of dystrophic skeletal muscle. I observed a significant reduction of muscle fiber necrosis in both TA and Dia muscles of *mdx* mouse, with a large decrease in the Dia muscle. This is of particular importance, because, the level of necrosis in untreated Dia muscle is higher compared to that in untreated TA muscle.[154]

The lower muscle fiber regeneration observed in RAPA-treated muscles may be a direct result of the reduction in muscle fiber necrosis, because a lower level of necrosis could diminish the need for muscle tissue regeneration. In addition, RAPA may inhibit protein synthesis in the treated muscle tissue.[155] Previous work suggests, however, that when mTOR is selectively knocked out in skeletal muscles, muscle fiber regeneration persists in affected muscles.[156]

A previous study of mTOR activation in the adult *mdx* mouse correlates advancing age with a reduction in mTOR signaling in *mdx* muscle between 18 and 24 months of age.[144] However, this previously reported study did not compare *mdx* with age-matched wild-type B10 mice. I hypothesized that mTOR activation would be altered in dystrophic muscle because of ongoing muscle fiber degeneration and regeneration that characterizes the pathologic process of muscular dystrophy. Furthermore, mTOR has been shown to be a crucial regulator of protein synthesis, which is required for effective muscle fiber regeneration. In this study, I observed that mTOR activation in Dia muscles of 6-week-old and 12-week-old *mdx* versus age-matched wild-type B10 mice is significantly different at both time points. mTOR activation is significantly higher in *mdx* mice at 6 weeks of age, when the dystrophic muscle fibers are undergoing a high level of regeneration. At 12 weeks of age, when the pathological processes of degeneration and regeneration have decrease in *mdx* dystrophic muscle tissue, however, I observed a significantly lower level of mTOR activation in Dia muscles of *mdx* mice compared to age-matched wild-type mice. In fact, mTOR activation increases by age in Dia muscle of B10 mice, but not in *mdx* mice, raising the possibility that the early reduction in muscle fiber regeneration that is observed in Dia muscles of *mdx* mice[157] could be related to a failure of mTOR activity to increase. In contrast to Dia muscle, mTOR activation was not significantly different in the TA muscle of dystrophic and wild-type mice at either 6- or 12-weeks of age. This suggests a difference between the patterns of

mTOR activation in TA versus Dia muscles of *mdx* mice. This is a novel finding that contributes to our understanding of the differences in function and progression of pathology between Dia and limb muscle of *mdx* mice.[158-160]

In addition to the differences observed in untreated *mdx* TA and Dia muscles, the findings of this study also show a significant difference between *mdx* TA and Dia muscles in response to treatment with the immunosuppressant drug RAPA. The data suggest that Dia muscle is more sensitive to the effects of RAPA treatment, supported by a more significant decrease in muscle fiber necrosis and a significant reduction in mTOR activation following RAPA treatment compared to TA muscles. Interestingly, in previous murine studies, which mTOR has been knocked out in skeletal muscles, Dia muscle showed a more severe pathological response with a higher level of muscle fiber damage to the absence of mTOR compared to other muscles.[156] It has also been shown in other studies that protein synthesis in rat hind-limb muscles can be independent of RAPA-sensitive pathways,[161] indicating that RAPA may not have the same effect in Dia and TA muscles. Additional studies of molecules in the mTOR pathway may further elucidate the differences between Dia and limb muscle in the *mdx* mouse.

In addition to the general effect of RAPA on muscle pathology in *mdx* mice, I have compared local versus systemic administration of RAPA on both disease pathology and mTOR activation. Local RAPA administration has been examined in studies that are relevant to induction of tolerance in organ transplantation[162, 163] and the systemic route of RAPA administration has been tested in studies of cancer[164] and aging.[165] My findings suggest that although there were more significant differences in lowering effector T cell infiltration and muscle fiber necrosis when RAPA was give systemically compared to when it was given locally, at least for the six-week duration of my studies, the positive effects were comparable between the

two administration strategies. Nonetheless, it is important to realize the negative effects of each strategy, as a systemic treatment affects tissues other than muscles and local treatment is more difficult to perform because it requires multiple and repeated injections. Provided the beneficial effects of the systemic and local RAPA treatments are comparable, in a disease such as DMD, in which every muscle tissue is affected, a systemic treatment may provide a more beneficial outcome. However, local RAPA treatment may find greater clinical applicability if preservation of individual muscles could improve quality of life of DMD patients.

In conclusion, the results of the present study, demonstrating the effect of RAPA on decreasing inflammation, preserving $Foxp3^+$ T cells, and decreasing necrosis in dystrophic *mdx* muscle tissue, could lead to the further development of treatments for DMD. In addition, the findings of novel differences of mTOR activation between TA and Dia muscles in *mdx* mice, both untreated and with RAPA treatment, add to the molecular understanding of the dystrophic phenotype.

4.0 HOST IMMUNOLOGICAL TOLERANCE TO HC-AD VECTOR MEDIATED DYSTROPHIN CDNA TRANSFER BY LOCAL IMMUNE SUPPRESSION.

4.1 RATIONALE

The following study was conducted to test the hypothesis that treatment of the *mdx* mouse muscles with RAPA prior to intramuscular HC-Ad vector-mediated dystrophin cDNA transfer will result in a decreased level of the host-anti-dystrophin immune responses and a long-term vector-mediated dystrophin protein expression in treated muscles.

4.2 INTRODUCTION

The host immune system is a major obstacle to successful transfer of a full-length dystrophin cDNA to dystrophic muscles of the *mdx* mouse model of DMD. Immune suppression in a dystrophic host may prevent host immunity against recombinant dystrophin protein and vector particles. However, systemic immune suppression can cause many side effects. One important side effect is an increased vulnerability to infectious pathogens and tumor growth. Therefore, it is important to find ways to suppress the immune system locally at a desired site of transgene expression, for instance, to avoid the negative consequences of a systemic down-regulation of host immunity.

One approach for the delivery of a chemical or a drug to an organ or tissue locally is the use of biologically degradable micro-particles that carry the desired drug to the target tissue. Injection of such micro-particles to the site of interest is the most common way to achieve site-specific delivery. Advantages of this approach include slow release of the drug and thus avoiding multiple injections in a short period of time. One example of using beads for immunosuppressant drug delivery is in studies of autoimmunity and organ or tissue transplantation.[166, 167] As an analogous approach to promote the success of dystrophin gene transfer, I hypothesized that RAPA-carrying beads would suppress anti-dystrophin immunity in dystrophic *mdx* muscle tissue treated with dystrophin vector delivery.

Therefore, the aim of the following study was to suppress the *mdx* mouse immune system only in TA muscles that receive dystrophin-carrying high capacity adenoviral (HC-Ad) vector to induce immune suppression and prevent specific immune responses to vector antigens and transgene product at the site of dystrophin protein expression. The PLGA micro-particles used in my study were relatively large with an average diameter of about 15 μm (compared to smaller forms that are about 4 μm in diameter). Therefore, I did not expect them to move in and out of the muscle tissue freely or to be easily picked up by circulating DCs, thus achieving a local treatment.

I used adult 6-week-old *mdx* mice in this study to assure that the host immune system was fully developed at the time of treatment. Six weeks following RAPA and dystrophin vector treatments mouse sera were examined for anti-dystrophin antibody production by the host and vector-mediated dystrophin expression was also evaluated in treated muscles.

4.3 METHODS

Mice: Wild type (C57BL/10J) and *mdx* (C57BL/10ScSnDmd*mdx*/J) mice were obtained from The Jackson Laboratory (Bar Harbor, ME).

Total muscle protein extract (TMPE) preparation: Freshly isolated mouse muscle was cut in small pieces in TEES buffer (50mM Tris-HCl pH 8.0, 5mM EGTA pH 7.4, 5mM EDTA pH 8.0, 5% SDS) and incubated on ice for 45 minutes. Samples were then sonicated briefly and centrifuged at 14,000 rpm for 20 minutes at 4°C. Supernatant was collected and stored at -80°C.

Rapamycin-containing PLGA microparticle preparation: RAPA microparticles were produced as described.[145] In brief, RAPA microparticles were produced using the single emulsion-evaporation technique that involved forming an emulsion of an organic solution containing rapa and PLGA in a bulk aqueous solution, through high speed homogenization (3000 rpm). Following evaporation of the organic solvent (dichloromethane), microparticles formed in the aqueous solution were freeze-dried and used in my experiments.

Intramuscular microparticle injection: Six-week-old *mdx* mice were injected intramuscularly with rapamycin-containing microparticles (RAPA beads) in sterile PBS (30µl per TA). Control mice either received empty microparticles (blank beads) or were left untreated.

Gel electrophoresis and Western blotting: This step was done according to standard protocols. The membranes with immobilized murine dystrophin protein used for the assay were generated from wild-type C57BL/10J (B10) total muscle protein extracts (TMPE) electrophoresed on SDS-PAGE. In brief, TMPE from B10 mice were run on 5% Acrylamide gel (Bio-Rad; Hercules, CA) for 3 hours at 110V. Protein samples were transferred from the gel to nitrocellulose membrane (Amersham biosciences) for 1.5 hours at 110V at 4°C. The membrane was blocked in 5% milk/1% sheep serum/TBST (10mM Tris pH 8.0, 0.15M NaCl, 0.5mM

Tween-20) overnight at 4°C. The membrane was then cut in pieces with a dystrophin protein band in each piece. A set dilution of the sera was used to examine the present or absence of an anti-dystrophin response. The pieces of membrane were then each incubated with a diluted serum sample (1:300) in TBST for 1.5 hours, then with HRP-conjugated sheep anti-mouse IgG (GE Healthcare; Piscataway, NJ) diluted in TBST for 45 minutes at room temperature. ECL detection reagent (GE Healthcare; Piscataway, NJ) was used to detect the chemiluminescent signal and Kodak film was used for visualizing the signal. The initial exposure time was 15-30 minutes and to confirm the absence of a band in irradiated mice an additional film was exposed to the membranes overnight.

Muscle Tissue Processing: Freshly dissected muscle was incubated in 2% paraformaldehyde/PBS on ice for 2 hours, then transferred into 30% sucrose overnight at 4°C. The next day the muscle tissue was snap-frozen in 2-methylbutane cooled with dry ice and stored at -80°C.

Immunohistochemistry of inflammatory cells: 10μm cryo-sections of muscle samples were prepared. Sections were rehydrated in PBS, blocked in peroxidase blocking reagent (DAKO Cytomation; Carpinteria, CA) for 5 minutes, and then blocked in 10% goat serum/PBS for 1 hour at room temperature. The primary antibody incubation using rat anti-CD4 and Foxp3 (ebiosciences; San Diego, CA) and rat anti-CD8 (Pharmingen; San Jose, CA) purified antibodies diluted in 10% goat serum/PBS were done for 1.5 hours at room temperature. Sections were incubated for 1 hour with secondary biotinylated goat anti-rat IgG (Pharmingen; San Jose, CA) diluted in DAKO antibody diluent (DAKO Cytomation; Carpinteria, CA). Sections were incubated in ABC Vectastain avidin-HRP detection solution (Vector Laboratories; Burlingame, CA) for 30 minutes at room temperature and DAB peroxidase substrate solution (Vector

Laboratories; Burlingame, CA) for 4 minutes. Eosin counterstaining was performed for visualization of muscle fibers. To analyze infiltrating cells in each group, the total number of cells per cross-section of vector-injected TA muscles was counted and the average number of cells from sections from different mice was calculated.

Statistical Analysis: In all performed studies the statistical analysis was performed by student's t-test, in which a treatment group and a control group, or two treatment groups were compared as unpaired sets. Values of variables were presented as the mean with standard deviation (SD). In all experiments, P values <0.05 were considered significant.

4.4 RESULTS

4.4.1 RAPA treatment does not prevent anti-dystrophin antibody production following vector-mediated dystrophin expression in *mdx* muscles

In order to examine if injection of RAPA-containing beads into *mdx* muscle could suppress humoral host immunity against vector-mediated dystrophin expression in the treated tissue, I examined the sera from RAPA-treated, vector-injected mice for production of anti-dystrophin antibodies. I compared the sera from treated mice to sera from mice that had received blank bead injections prior to an intramuscular dystrophin vector injection in the TA muscles. To assess production of anti-dystrophin antibodies in the sera of the treated mice I prepared total muscle protein extracts from wild-type C57BL/10 mice that do produce dystrophin protein in their muscle tissue, and immobilized that on a nitrocellulose membrane, which was then incubated with sera from the treated mice. A secondary anti-mouse IgG was used to detect any

mouse antibodies that may have been bound to the membrane-bound muscle proteins, including dystrophin protein.

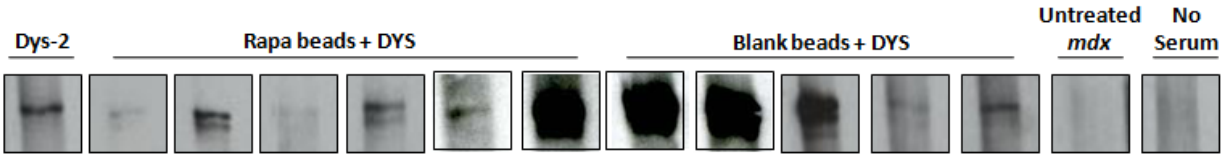

Figure 21: Anti-dystrophin humoral response in mice that had received RAPA or blank microparticle injections prior to dystrophin vector injection. Mice were treated at 6 weeks of age and dystrophin vector was injected into TA muscles of RAPA or blank bead-injected mice at about 24 hours following bead-injections. Mice were sacrificed and treated muscle tissue was collected 6 weeks post-treatment. Sera were analyzed for anti-dystrophin antibody production. A monoclonal anti-dystrophin antibody (Dys-2) was used as a positive control. Negative controls were sera from untreated age-matched *mdx* mouse and a sample with no serum incubation (secondary staining, only).

Surprisingly, I observed that all of the mice that had received RAPA treatment prior to dystrophin vector transfer produced anti-dystrophin antibodies (Fig. 21). The mice that had been injected with blank beads also produced strong anti-dystrophin antibody, as expected, and at higher levels compared to the mice that received RAPA treatment and dystrophin vector injection. This observation suggested that local RAPA treatment did not lead to a complete elimination of host immune response against vector-mediated dystrophin protein expression in the treated muscle tissue, as all treated mice did produce anti-dystrophin antibody following vector-mediated dystrophin expression. Nonetheless, RAPA treatment down-regulated host immunity against dystrophin protein by lowering the level of anti-dystrophin antibody production compared to the blank bead-treatment in dystrophin vector-injected *mdx* mice.

4.4.2 RAPA treatment reduces infiltration of effector T cells, but not regulatory T cells in muscle

To examine the effect of RAPA on T cell infiltration following vector-mediated dystrophin expression, levels of $CD4^+$, $CD8^+$, and $Foxp3^+$ cells were evaluated in TA muscles of *mdx* mice treated with RAPA or blank beads prior to dystrophin vector injection. T cell infiltration was analyzed through immunohistochemistry and average cell numbers per TA cross-section were compared. $CD4^+$ T cells were significantly lower in RAPA bead-treated, vector-injected and age-matched untreated *mdx* TA muscles compared to blank bead-treated, vector-injected TA muscles (Fig. 22). $CD8^+$ T cells were also at lower levels in both RAPA bead-treated, vector-injected and untreated *mdx* muscles compared to blank bead-treated, vector-injected muscles, but the difference was only significant between untreated and blank bead-treated groups. The difference in $CD8^+$ T cells between RAPA bead-treated and blank bead-treated groups did not reach significance (p=0.13). Interestingly, however, the level of Foxp3+ T

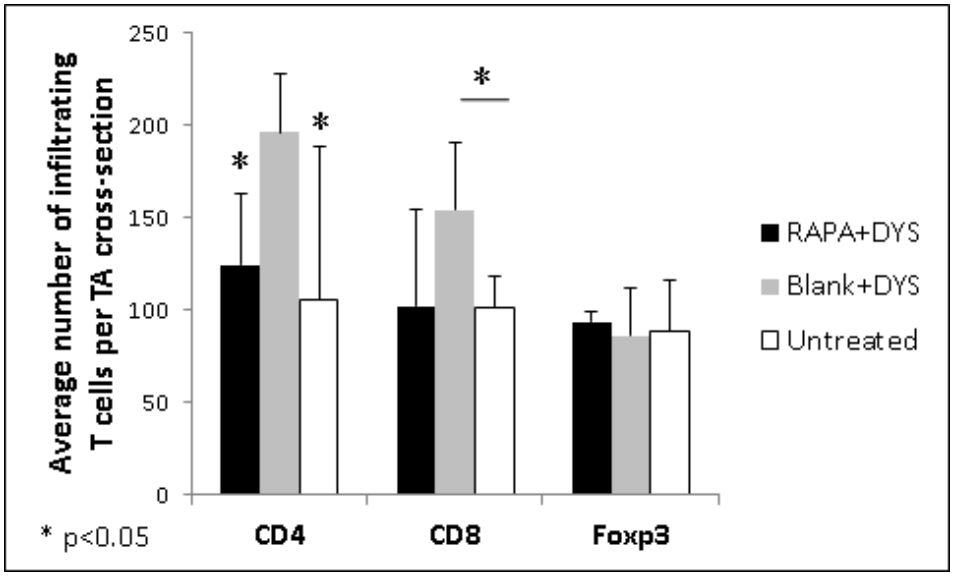

Figure 22: T cell infiltration in RAPA bead- or blank bead-treated, dystrophin vector-injected *mdx* TA muscles. Six weeks following treatment TA muscles were analyzed for T cell infiltration following vector-mediated dystrophin expression. T cells were detected through immunohistochemistry staining and were counted and averaged per TA cross-section. n=6 for RAPA bead-injected and n=4 for blank bead-injected mice. * indicates p value < 0.05.

cells was not lower in RAPA bead-treated muscles compared to blank bead-treated or untreated muscles (Fig. 22).

4.4.3 RAPA treatment is not associated with a higher level of vector-mediated DYS expression in *mdx* muscles

Through immunofluorescence staining for dystrophin protein in muscle cross-sections, I observed vector-mediated dystrophin expression in RAPA bead-treated muscles that was higher than the background level of dystrophin expression in revertant fibers in untreated age-matched muscles (Fig. 23).

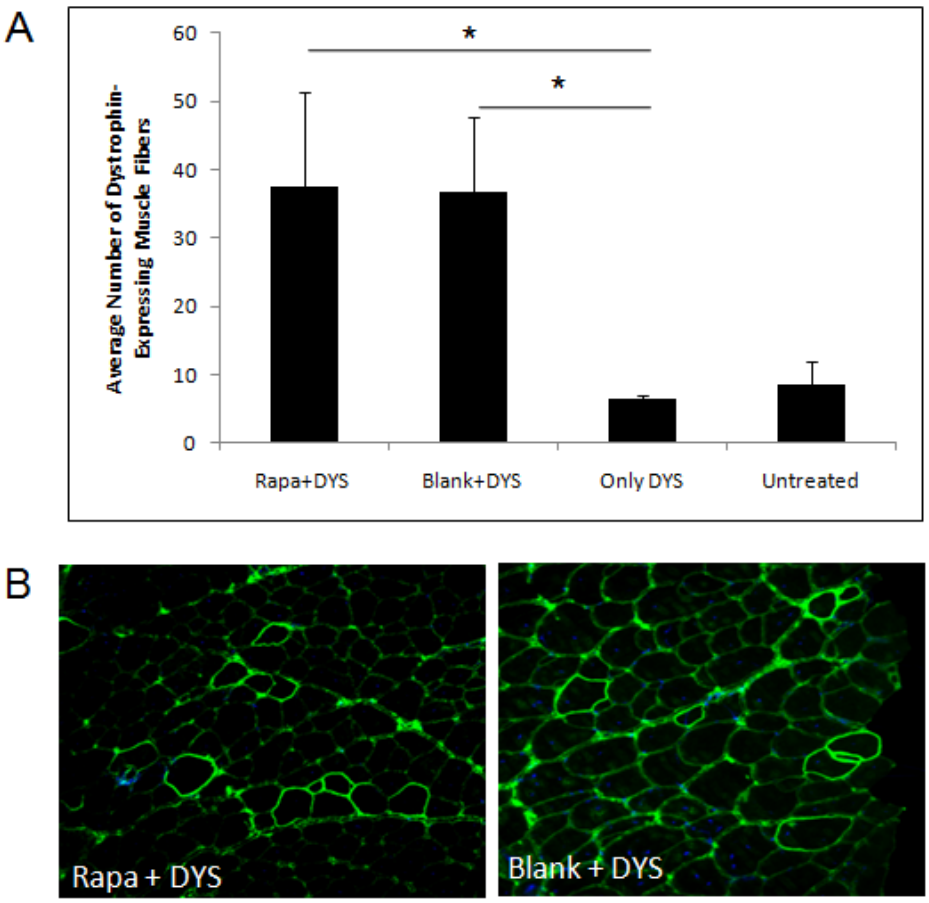

Figure 23: Vector-mediated dystrophin protein expression in RAPA bead- or blank bead-treated *mdx* TA muscles. Six weeks following treatment TA muscles were analyzed for dystrophin protein expression. Dystrophin-expressing fibers were counted on cross-sections of TA muscles from various longitudinal positions on each TA muscle and were averaged per TA section (A). Detection of dystrophin-expressing fibers was done by immuno-fluorescent staining using a monoclonal anti-dystrophin primary antibody (Dys-2) and a fluorescent-conjugated secondary antibody for visualization of the dystrophin-expressing fibers (B).

Nonetheless, surprisingly, the mice that were injected with blank beads and dystrophin vector showed a comparable but slightly higher level of dystrophin expression at 6 weeks following treatment (Fig. 23).

4.5 DISCUSSION

Evasion of the host-anti-dystrophin immune response is required for successful therapeutic gene replacement in DMD patients as well as in animal models of DMD including the *mdx* mouse.[8, 9, 168-170] Therefore, down-regulation of the host immune system prior to and during vector transfer and vector-mediated gene expression is an important factor in finding a practical approach to transfer a healthy dystrophin gene into muscles of the dystrophic host.

The immunosuppressive drug, RAPA, has been successfully used in other applications, including organ and tissue transplantation, in which suppression of immunity is desired.[147, 171] A wide range of transplantation studies, from lung to bone marrow transplantations, have showed beneficial effects of using RAPA to reduce or eliminate both graft-versus-host and host-versus-graft responses that are common in transplanted patients. Mechanisms of action include inhibition of effector T cell activation and proliferation, Treg expansion, or prevention of cytotoxic cytokine production.[172-174] Considering the beneficial advantages that RAPA has in down-regulating host immunity against neo-antigens in organ and tissue transplantation, I pursued the investigations presented in this section to see if the effects of RAPA on the immune system of the *mdx* host may benefit vector-mediated expression of dystrophin.

Although RAPA is a potent immuosuppressive agent, other effects of the drug may limit its utility. It was previously reported that RAPA may prevent protein expression through binding

to its target protein, mTOR in mammals, which is a major regulator of cell growth and protein synthesis in response to energy and nutrients.[175-177] Therefore, it is considered that the immunosuppressive effects of RAPA and its inhibitory effects on protein expression could have opposite effects on vector-mediated dystrophin expression in *mdx* muscles. This may explain why RAPA-treated, vector-injected *mdx* muscles did not show a higher level of vector-mediated dystrophin expression.

Nonetheless, data presented in this study clearly showed that the immunosuppressive characteristic of RAPA indeed had a positive effect on decreasing antibody production in dystrophin vector-injected mice, compared to the blank bead-injected, dystrophin vector-injected mice. I observed that although all RAPA- and blank-bead-treated *mdx* mice developed an anti-dystrophin antibody response following vector-mediated dystrophin expression, RAPA-treated mice showed a significant reduction of the anti-dystrophin antibody response.

Furthermore, similar to the effect of RAPA on lowering host humoral immunity against vector-mediated dystrophin protein expression, T cell infiltration in RAPA bead-treated *mdx* muscle tissue was also affected. As shown previously, vector-mediated dystrophin expression in *mdx* muscles leads to effector T cell infiltration at levels significantly higher than untreated *mdx* muscles.[178] In agreement to the previous findings, I show here that when the mice were treated with blank beads prior to dystrophin vector transfer, a significantly higher level of $CD4^+$ and $CD8^+$ T cells infiltrate in the treated muscle tissue. Nonetheless, RAPA treatment prior to dystrophin vector transfer leads to infiltration of $CD4^+$ and $CD8^+$ T cells at levels comparable to the untreated *mdx* muscles, indicating the positive effect of RAPA on down-regulating host cellular immunity following vector-mediated dystrophin expression.

In addition to the effects of RAPA on vector-mediated gene expression, a slight effect of the RAPA-encapsulated PLGA beads in the injected muscle tissue has to be considered as well, as the PLGA beads are acidic, therefore, affecting the pH of the injected site. Previous studies have indicated that lowering the pH may promote gene expression, as one study showed 2-14 fold increase in protein synthesis when pH was lowered from 7.0 to 5.0 in bacterial systems.[179] Therefore, the effect of the beads on the pH of the injected muscle tissue is an important factor in analysis of the presented findings. My data shows that RAPA beads and blank beads give comparable results in terms of vector-mediated dystrophin expression; they both lead to significantly higher level of dystrophin expression in the injected muscle tissues. In fact, vector-mediated dystrophin expression appears slightly lower in the *mdx* muscles that had received RAPA beads compared to those receiving only blank beads. Possible explanation for this observation is that the slightly acidic nature of the beads promotes successful vector-mediated gene expression, while RAPA prevents gene expression in the injected muscle tissue.

Overall, data shown here indicate that regardless of its immunosuppressive effects in treated tissues in cases such as organ or tissue transplantation, RAPA may not be a beneficial adjunct to vector-mediated gene therapy that depends on expression of the recombinant gene in addition to successful gene delivery and persistence.

5.0 CONCLUSIONS AND IMPLICATIONS

Recent studies on DMD have extensively focused on better understanding the disease[2, 22, 24, 119, 180-182] and evaluating potential therapeutic approaches[4, 5, 7, 9, 10, 36, 52, 183] to cure this devastating lethal disorder. A clear molecular knowledge on the mechanism of the disease is crucial for enabling us to find the best possible treatment for DMD. In addition, evaluation of potential barriers for successful treatment and understanding the mechanisms behind these barriers is equally important toward a goal of ameliorating the disease phenotype. In the work presented here, the major goal has been to assess immunological problems that are associated with both dystrophin gene therapy and the disease-associated pathology. To accomplish these aims, I used irradiation to test the effect of a temporal elimination of immunity as an adjunct to gene transfer. I also studied the effects of the immunosuppressive drug, RAPA, on both untreated and dystrophin vector-injected *mdx* muscles.

5.1 IRRADIATION FOR GENE THERAPY

Irradiation has been widely used for down-regulation of undesired immunity in mice.[50, 184-188] In my studies, I observed that irradiation can delay and reduce host immunity against vector-mediated dystrophin expression in adult *mdx* mice; an observation that was not reported previously. Through this irradiation-mediated transient removal of the host immune system, I

was able to temporally analyze the developing anti-dystrophin host immune response. The findings of this aim are particularly important in understanding various aspects of host anti-dystrophin immune response in dystrophic mice, with an ultimate goal of a better understanding of an anti-dystrophin immune response in DMD patients.

5.2 RAPAMYCIN IN DYSTROPHIC MUSCLE

Immune suppression is currently considered one of the common treatments for a DMD patient.[34, 37, 38, 189, 190] Through analyzing the immunosuppressive effects of RAPA on dystrophic *mdx* muscles, I learned that although RAPA may have negative effects on cell growth and protein synthesis, it has significant benefits on dystrophic muscle phenotype and has the potential to be considered as a beneficial treatment for DMD patients suffering from severe muscle fiber necrosis and T cell infiltration in their muscle tissue. As a valuable consequence of treating *mdx* mice with RAPA, I also observed that there are major differences in mTOR activation between TA and diaphragm muscles in *mdx* mice with and without RAPA treatment. This finding is novel and requires a more in-depth analysis of the mTOR pathway in dystrophic muscle. The results of this study are important for future work on both therapeutic and molecular aspects of DMD.

5.3 RAPAMYCIN FOR GENE THERAPY

Since RAPA has been successfully used in down-regulating host immunity in organ and tissue transplantation,[134, 151, 167, 172] I conducted studies, in which RAPA was used to down-

regulate anti-dystrophin immunity in dystrophin vector-injected *mdx* mice. Aside from the positive therapeutic effects that RAPA seems to have for treating dystrophic muscles, it may not be a beneficial candidate for using as an immunosuppressive drug along with gene delivery in *mdx* muscles. In fact, the characteristic of RAPA that interferes with protein synthesis seems to directly affect vector-mediated dystrophin expression, and therefore does not allow for successful therapeutic gene replacement in these mice.

Overall, I expect that the studies presented here provide beneficial data on the immunity aspects of both therapeutic gene replacement in dystrophic muscle, and DMD phenotype. Of course, more questions will follow the findings explained in this thesis that will require further in-depth investigation. Potential future studies may deal with a further analysis of 1) the differences observed between peripheral and central anti-dystrophin host immune responses following vector-mediated dystrophin expression in *mdx* muscles, 2) the activities of proteins upstream and downstream of mTOR in dystrophic muscles, 3) the effects of immunosuppressive drugs other than RAPA on dystrophin gene transfer in the *mdx* mouse, and other additional questions that can shed more light on each of these matters. At this point, I hope that the results of the studies presented here will contribute toward the quest to find a cure for DMD patients.

6.0 BIBLIOGRAPHY

(1) Drousiotou A, Ioannou P, Georgiou T, Mavrikiou E, Christopoulos G, Kyriakides T, et al. Neonatal screening for Duchenne muscular dystrophy: a novel semiquantitative application of the bioluminescence test for creatine kinase in a pilot national program in Cyprus. *Genet Test* 1998;**2**(1):55-60.

(2) Emery AE. Population frequencies of inherited neuromuscular diseases--a world survey. *Neuromuscul Disord* 1991;**1**(1):19-29.

(3) Parsons EP, Bradley DM, Clarke AJ. Newborn screening for Duchenne muscular dystrophy. *Arch Dis Child* 2003 Jan;**88**(1):91-92.

(4) Alba R, Bosch A, Chillon M. Gutless adenovirus: last-generation adenovirus for gene therapy. *Gene Therapy* 2005 Jun 1;**12**:18-27.

(5) Gilbert R, Nalbantoglu J, Howell JM, Davies L, Fletcher S, Amalfitano A, et al. Dystrophin expression in muscle following gene transfer with a fully deleted ("gutted") adenovirus is markedly improved by trans-acting adenoviral gene products. *Hum Gene Ther* 2001 Sep 20;**12**(14):1741-1755.

(6) Jiang Z, Schiedner G, Gilchrist S, Kochanek S, Clemens PR. CTLA4Ig delivered by high-capacity adenoviral vector induces stable expression of dystrophin in *mdx* mouse muscle. *Gene Therapy* 2007 Oct;**11**(19):1453-1461.

(7) Acsadi G, Lochmuller H, Jani A, Huard J, Massie B, Prescott S, et al. Dystrophin expression in muscles of *mdx* mice after adenovirus-mediated in vivo gene transfer. *Hum Gene Ther* 1996 Jan 20;**7**(2):129-140.

(8) Ferrer A, Wells KE, Wells DJ. Immune responses to dystropin: implications for gene therapy of Duchenne muscular dystrophy. *Gene Ther* 2000 Sep;**7**(17):1439-1446.

(9) Gilchrist S, Ontel M, Kochanek S, Clemens PR. Immune response to full-length dystrophin delivered to Dmd muscle by a high-capacity adenoviral vector. *Mol Ther* 2002 Sep;**6**(3):359-368.

(10) Jiang Z, Schiedner G, van Rooijen N, Liu CC, Kochanek S, Clemens PR. Sustained Muscle Expression of Dystrophin from a High-Capacity Adenoviral Vector with Systemic Gene Transfer of T Cell Costimulatory Blockade. *Mol Ther* 2004 Oct;**10**(4):688-696.

(11) Koppanati BM, Li J, Reay DP, Wang B, Daood M, Zheng H, et al. Improvement of the *mdx* mouse dystrophic phenotype by systemic in utero AAV8 delivery of a minidystrophin gene. *Gene Ther* 2010 Jun 10.

(12) Koppanati BM, Li J, Xiao X, Clemens PR. Systemic delivery of AAV8 in utero results in gene expression in diaphragm and limb muscle: treatment implications for muscle disorders. *Gene Ther* 2009 Sep;**16**(9):1130-1137.

(13) Reay DP, Bilbao R, Koppanati BM, Cai L, O'Day TL, Jiang Z, et al. Full-length dystrophin gene transfer to the *mdx* mouse in utero. *Gene Ther* 2008 Apr;**15**(7):531-536.

(14) Bilbao R, Reay DP, Li J, Xiao X, Clemens PR. Patterns of gene expression from in utero delivery of adenoviral-associated vector serotype 1. *Hum Gene Ther* 2005 Jun;**16**(6):678-684.

(15) Bilbao R, Reay DP, Wu E, Zheng H, Biermann V, Kochanek S, et al. Comparison of high-capacity and first-generation adenoviral vector gene delivery to murine muscle in utero. *Gene Ther* 2005 Jan;**12**(1):39-47.

(16) Farini A, Meregalli M, Belicchi M, Battistelli M, Parolini D, D'Antona G, et al. T and B lymphocyte depletion has a marked effect on the fibrosis of dystrophic skeletal muscles in the scid/*mdx* mouse. *Journal of Pathology* 2007 Jul 31;**213**:229-238.

(17) Spencer MJ M-REDKTJG. Helper (CD4(+)) and cytotoxic (CD8(+)) T cells promote the pathology of dystrophin-deficient muscle. *Clinical Immunology* 2001 Feb;**98**(2):235-243.

(18) Spencer MJ, Tidball JG. Do immune cells promote the pathology of dystrophin-deficient myopathies? *Neuromuscul Disord* 2001 Sep;**11**(6-7):556-564.

(19) Emery AE. Dystrophin function. *Lancet* 1990 May 26;**335**(8700):1289.

(20) Hoffman EP, Brown RH, Jr., Kunkel LM. Dystrophin: the protein product of the Duchenne muscular dystrophy locus. *Cell* 1987 Dec 24;**51**(6):919-928.

(21) Love DR, Davies KE. Duchenne muscular dystrophy: the gene and the protein. *Mol Biol Med* 1989 Feb;**6**(1):7-17.

(22) Allen DG, Gervasio OL, Yeung EW, Whitehead NP. Calcium and the damage pathways in muscular dystrophy. *Can J Physiol Pharmacol* 2010 Feb;**88**(2):83-91.

(23) Fanchaouy M, Polakova E, Jung C, Ogrodnik J, Shirokova N, Niggli E. Pathways of abnormal stress-induced Ca2+ influx into dystrophic *mdx* cardiomyocytes. *Cell Calcium* 2009 Aug;**46**(2):114-121.

(24) Gissel H. The role of Ca2+ in muscle cell damage. *Ann N Y Acad Sci* 2005 Dec;**1066**:166-180.

(25) Phillips MF, Quinlivan R. Calcium antagonists for Duchenne muscular dystrophy. *Cochrane Database Syst Rev* 2008;(4):CD004571.

(26) Lewis C, Carberry S, Ohlendieck K. Proteomic profiling of x-linked muscular dystrophy. *J Muscle Res Cell Motil* 2009 Dec;**30**(7-8):267-269.

(27) Gowers WR. Clinical lectures on pseudohypertrophic muscular paralysis. *Lancet* 1879;**2**:73-75.

(28) Shaikhly KI, Hamdan FB, Al-Ani FS. Power spectrum analysis and conventional electromyogram in Duchenne muscular dystrophy. *Saudi Med J* 2000 Nov;**21**(11):1038-1042.

(29) Tanabe Y, Esaki K, Nomura T. Skeletal muscle pathology in X chromosome-linked muscular dystrophy (*mdx*) mouse. *Acta Neuropathol* 1986;**69**(1-2):91-95.

(30) Bulfield G, Siller W, Wight P, Moore K. X Chromosome-linked muscular dystrophy (*mdx*) in the mouse. *Proc Natl Acad Sci* 1984 Feb 15;**81**:1189-1192.

(31) Chamberlain JS, Farwell NJ, Chamberlain JR, Cox GA, Caskey CT. PCR analysis of dystrophin gene mutation and expression. *J Cell Biochem* 1991 Jul;**46**(3):255-259.

(32) Chamberlain JS, Phelps SF, Cox GA, Maichele AJ, Greenwood AD. PCR analysis of muscular dystrophy in *mdx* mice. *Mol Cell Biol Hum Dis Ser* 1993;**3**:167-189.

(33) Sicinski P, Geng Y, Ryder-Cook AS, Barnard EA, Darlison MG, Barnard PJ. The molecular basis of muscular dystrophy in the *mdx* mouse: a point mutation. *Science* 1989 Jun 30;**244**(4912):1578-1580.

(34) Bach JR, Martinez D, Saulat B. Duchenne muscular dystrophy: the effect of glucocorticoids on ventilator use and ambulation. *Am J Phys Med Rehabil* 2010 Aug;**89**(8):620-624.

(35) Hussein M, Hamed S, Mostafa M, Abu-Dief E, Kamel N, Kandil M. The effects of glucocorticoid therapy on the inflammatory and dendritic cells in muscular dystrophies. *Ent J Exp Pathol* 2006 Dec 15;**87**(6):451-461.

(36) Merlini L, Cicognani A, Malaspina E, Gennari M, Gnudi S, Talim B, et al. Early prednisone treatment in Duchenne muscular dystrophy. *Muscle Nerve* 2003 Feb;**27**(2):222-227.

(37) Straathof CS, Overweg-Plandsoen WC, van den Burg GJ, van der Kooi AJ, Verschuuren JJ, de Groot IJ. Prednisone 10 days on/10 days off in patients with Duchenne muscular dystrophy. *J Neurol* 2009 May;**256**(5):768-773.

(38) Alman BA, Raza SN, Biggar WD. Steroid treatment and the development of scoliosis in males with duchenne muscular dystrophy. *J Bone Joint Surg Am* 2004 Mar;**86-A**(3):519-524.

(39) Cheuk DK, Wong V, Wraige E, Baxter P, Cole A, N'Diaye T, et al. Surgery for scoliosis in Duchenne muscular dystrophy. *Cochrane Database Syst Rev* 2007;(1):CD005375.

(40) Heller KD, Wirtz DC, Siebert CH, Forst R. Spinal stabilization in Duchenne muscular dystrophy: principles of treatment and record of 31 operative treated cases. *J Pediatr Orthop B* 2001 Jan;**10**(1):18-24.

(41) Farini A, Razini P, Erratico S, Torrente Y, Meregalli M. Cell based therapy for Duchenne muscular dystrophy. *J Cell Physiol* 2009 Dec;**221**(3):526-534.

(42) Payne TR, Oshima H, Sakai T, Ling Y, Gharaibeh B, Cummins J, et al. Regeneration of dystrophin-expressing myocytes in the *mdx* heart by skeletal muscle stem cells. *Gene Ther* 2005 Aug;**12**(16):1264-1274.

(43) Floyd SS, Jr., Clemens PR, Ontell MR, Kochanek S, Day CS, Yang J, et al. Ex vivo gene transfer using adenovirus-mediated full-length dystrophin delivery to dystrophic muscles. *Gene Ther* 1998 Jan;**5**(1):19-30.

(44) Moisset PA, Skuk D, Asselin I, Goulet M, Roy B, Karpati G, et al. Successful transplantation of genetically corrected DMD myoblasts following ex vivo transduction with the dystrophin minigene. *Biochem Biophys Res Commun* 1998 Jun 9;**247**(1):94-99.

(45) Fan Y, Maley M, Beilharz M, Grounds M. Rapid death of injected myoblasts in myoblast transfer therapy. *Muscle Nerve* 1996 Jul;**19**(7):853-860.

(46) Hodgetts SI, Beilharz MW, Scalzo AA, Grounds MD. Why do cultured transplanted myoblasts die in vivo? DNA quantification shows enhanced survival of donor male myoblasts in host mice depleted of CD4+ and CD8+ cells or Nk1.1+ cells. *Cell Transplant* 2000 Jul;**9**(4):489-502.

(47) Sammels LM, Bosio E, Fragall CT, Grounds MD, van RN, Beilharz MW. Innate inflammatory cells are not responsible for early death of donor myoblasts after myoblast transfer therapy. *Transplantation* 2004 Jun 27;**77**(12):1790-1797.

(48) Camirand G, Caron NJ, Asselin I, Tremblay JP. Combined immunosuppression of mycophenolate mofetil and FK506 for myoblast transplantation in *mdx* mice. *Transplantation* 2001 Jul 15;**72**(1):38-44.

(49) Vilquin JT, Kinoshita I, Roy R, Tremblay JP. Cyclophosphamide immunosuppression does not permit successful myoblast allotransplantation in mouse. *Neuromuscul Disord* 1995 Nov;**5**(6):511-517.

(50) Hodgetts SI, Grounds MD. Irradiation of dystrophic host tissue prior to myoblast transfer therapy enhances initial (but not long-term) survival of donor myoblasts. *J Cell Sci* 2003 Oct 15;**116**(Pt 20):4131-4146.

(51) Koppanati BM, Li J, Xiao X, Clemens PR. Systemic delivery of AAV8 in utero results in gene expression in diaphragm and limb muscle: treatment implications for muscle disorders. *Gene Ther* 2009 Sep;**16**(9):1130-1137.

(52) Koppanati BM, Li J, Reay DP, Wang B, Daood M, Zheng H, et al. Improvement of the *mdx* mouse dystrophic phenotype by systemic in utero AAV8 delivery of a minidystrophin gene. *Gene Ther* 2010 Jun 10.

(53) Smith-Arica JR, Bartlett JS. Gene therapy: recombinant adeno-associated virus vectors. *Curr Cardiol Rep* 2001 Jan;**3**(1):43-49.

(54) Duan D, Yue Y, Engelhardt JF. Expanding AAV packaging capacity with trans-splicing or overlapping vectors: a quantitative comparison. *Mol Ther* 2001 Oct;**4**(4):383-391.

(55) Duan D, Yue Y, Engelhardt JF. Dual vector expansion of the recombinant AAV packaging capacity. *Methods Mol Biol* 2003;**219**:29-51.

(56) Samulski RJ. Expanding the AAV package. *Nat Biotechnol* 2000 May;**18**(5):497-498.

(57) Mays LE, Vandenberghe LH, Xiao R, Bell P, Nam HJ, Agbandje-McKenna M, et al. Adeno-associated virus capsid structure drives CD4-dependent CD8+ T cell response to vector encoded proteins. *J Immunol* 2009 May 15;**182**(10):6051-6060.

(58) Mingozzi F, High K. Immune responses to AAV in clinical trials. *Curr Gene Ther* 2007 Oct 15;**7**(5):316-324.

(59) Mingozzi F, Maus MV, Hui DJ, Sabatino DE, Murphy SL, Rasko JE, et al. CD8(+) T-cell responses to adeno-associated virus capsid in humans. *Nat Med* 2007 Apr;**13**(4):419-422.

(60) Wang Z, Allen JM, Riddell SR, Gregorevic P, Storb R, Tapscott SJ, et al. Immunity to adeno-associated virus-mediated gene transfer in a random-bred canine model of Duchenne muscular dystrophy. *Hum Gene Ther* 2007 Jan;**18**(1):18-26.

(61) Kochanek S, Clemens PR, Mitani K, Chen H, Chan S, Caskey C. A new adenoviral vector: Replacement of all viral coding secuences with 28 kb of DNA independently expressing both full-length dystrophin and beta-galactosidase. *Gene Therapy* 1996 Nov 1;**3**(11):965-972.

(62) Jiang Z, Fingold E, Kochanek S, Clemens PR. Systemic delivery of a high-capacity adenoviral vector expressing mouse CTLA4Ig improves skeletal muscle gene therapy. *Mol Ther* 2007;**6**(3):369-376.

(63) Zhou HS, Zhao T, Rao XM, Beaudet AL. Production of helper-dependent adenovirus vector relies on helper virus structure and complementing. *J Gene Med* 2002 Sep;**4**(5):498-509.

(64) Di Paolo NC, Shayakhmetov DM. Adenovirus de-targeting from the liver. *Curr Opin Mol Ther* 2009 Oct;**11**(5):523-531.

(65) Carl JW, Jr., Liu JQ, Joshi PS, El-Omrani HY, Yin L, Zheng X, et al. Autoreactive T Cells Escape Clonal Deletion in the Thymus by a CD24-Dependent Pathway. *J Immunol* 2008 Jul 1;**181**(1):320-328.

(66) Tarlinton DM, Batista F, Smith KG. The B-cell response to protein antigens in immunity and transplantation. *Transplantation* 2008 Jun 27;**85**(12):1698-1704.

(67) Chu D, Sullivan C, Weitzman M, Du L, Wolf P, Jamieson S, et al. Direct comparison of efficiency and stability of gene transfer into the mammalian heart using adeno-associated virus versus adenovirus vectors. *J Thorac Cardiovasc Surg* 2003 Sep 1;**126**(3):671-679.

(68) Jooss K, Yang Y, Fisher K, Wilson J. Transduction of dendritic cells by DNA viral vectors directs the immune response to transgene products in muscle fibers. *J Virol* 1998 Jun 1;**72**:4212-4223.

(69) Jooss K, Chirmule N. Immunity to adenovirus and adeno-associated viral vectors: implications for gene therapy. *Gene Therapy* 2003 Jun 1;**10**(11):955-963.

(70) Yuasa K, Yoshimura M, Urasawa N, Ohshima S, Howell J, Nakamura A, et al. Injection of recombinant AAV serotype 2 into canine skeletal muscles evokes strong immune responses against transgene productf. *Gene Therapy* 2007 Sep 15;**14**(17):1249-1260.

(71) Harvey B, Hackett N, El-Sawy T, Rosengart T, Hirschowitz E, Lieberman M, et al. Variability of human systemic humoral immune responses to adenovirus gene transfer vectors administered to different organs. *J Virol* 1999 Aug 1;**73**(8):6729-6742.

(72) Dobrzynski E, Herzog RW. Tolerace induction by viral in vivo gene transfer. *Clinical Medicine and Research* 2005 Nov 4;**3**:234-240.

(73) Jerebtsova M, Batshaw M, Ye X. Humoral immune response too recombinant adenovirus and adeno-associated virus after in utero administration of viral vectors in mice. *Pediatr Res* 2002 Jul 1;**52**(1):95-104.

(74) Xiao W, Chirmule N, Schnell M, Tazelaar J, Hughes J, Wilson J. Route of administration determines induction of T-cell-independent humoral responses to adeno-associated virus vectors. *Mol Ther* 2000 Apr 1;**1**(4):323-329.

(75) Dobrzynski E, Mingozzi F, Liu YL, Bendo E, Cao O, Wang L, et al. Induction of antigen-specific CD4+ T-cell anergy and deletion by in vivo viral gene transfer. *Blood* 2004 Aug 15;**104**(4):969-977.

(76) Mount JD, Herzog RW, Tillson D, Goodman S, Robinson N, McCleland M, et al. Sustained phenotypic correction of hemophilia B dogs with a factor IX null mutation by liver-directed gene therapy. *Blood* 2002 Jun 1;**99**:2670-2676.

(77) Kelly R, Alonso S, Tajbakhsh S, Cossu G, Buckingham M. Myosin light chain 3F regulatory sequences confer regionalized cardiac and skeletal muscle expression in transgenic mice. *J Cell Biol* 1995 Apr;**129**(2):383-396.

(78) Snyder RO, Miao CH, Patijn GA, Spratt SK, Danos O, Nagy D, et al. Persistent and therapeutic concentrations of human factor IX in mice after hepatic gene transfer of recombinant AAV vectors. *Nat Genet* 1997 Jul;**16**(3):270-276.

(79) Wang L, Nichols TC, Read MS, Bellinger DA, Verma IM. Sustained expression of therapeutic level of factor IX in hemophilia B dogs by AAV-mediated gene therapy in liver. *Mol Ther* 2000 Feb;**1**(2):154-158.

(80) Wang R, Han G, Song L, Chen G, Xu R, Yu M, et al. Foxp3-expressing CD4(+) T cells under the control of INF-gamma promoter prevent diabetes in NOD mice. *Mol Ther* 2007 Aug 1;**15**(8):1551-1557.

(81) Belkaid Y. Regulatory T cells and infection: a dangerous necessity. *Nature Reviews Immunology* 2007 Nov 1;**7**(11):875-888.

(82) Sakaguchi S. Regulatory T cells: key controllers of immunologic self-tolerance. *Cell* 2000 May 26;**101**(5):455-458.

(83) Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell* 2008 May 30;**133**(5):775-787.

(84) Brunkow M. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet* 2001;**27**:68-73.

(85) Appleby MW, Ramsdell F. Scurfy, the Foxp3 locus, and the molecular basis of peripheral tolerance. *Curr Top Microbiol Immunol* 2008;**321**:151-168.

(86) Del PG. The complexity of the CD4 T-cell responses: old and new T-cell subsets. *Parassitologia* 2008 Jun;**50**(1-2):9-16.

(87) Grossman W. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity* 2004;**21**:589-601.

(88) Barthlott T, Kassiotis G, Stockinger B. T cell regulation as a side-effect of homeostasis and competition. *J Exp Med* 2003;**197**:451-460.

(89) Vignali DAA, Collison LW, Workman CJ. How regulatory T cells work. *Nat Rev Immunol* 2008 Jul;**8**(7):523-532.

(90) Huang CT, Workman CJ, Flies D, Pan X, Marson AL, Zhou G, et al. Role of LAG-3 in Regulatory T Cells. *Immunity* 2004 Oct;**21**(4):503-513.

(91) Collison LW, Workman CJ, Kuo TT, Boyd K, Wang Y, Vignali KM, et al. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature* 2007 Nov 22;**450**(7169):566-569.

(92) Stephens L, Gray D, Anderton S. CD4+CD25+ regulatory T cells limit the risk of autoimmune disease arising from T cell receptor crossreactivity. *PNAS* 2005 Nov 29;**102**(48):17418-17423.

(93) Chai J, Xue S, Coe D, Addey C, Bartok I, Scott D, et al. Regulatory T cells, derived from naive CD4+CD25- T cells by in vitro Foxp3 gene transfer, can induce transplantation tolerance. *Transplantation* 2005 May 27;**79**(10):1310-1316.

(94) Raimondi G, Turner MS, Thomson AW, Morel PA. Naturally occurring regulatory T cells: recent insights in health and disease. *Crit Rev Immunol* 2007;**27**(1):61-95.

(95) Seveno C, Coulon F, Haspot F, Merieau E, Renaudin K, Martinet B, et al. Induction of regulatory cells and control of cellular but not vascular rejection by costimulation blockade in hamster-to-rat heart xenotransplantation. *Xenotransplantation* 2006 Aug 21;**14**:25-33.

(96) Fehervari Z, Sakaguchi S. Control of Foxp3+CD4+CD25+ regulatory cell activation and function by dendritic cells. *Japanese Society of Immunology* 2004 Nov 1;**16**(12):1769-1780.

(97) Bruder D, Probst-Kepper M, Westendorf AM, Geffers R, Beissert S, Loser K, et al. Neuropilin-1: a surface marker of regulatory T cells. *Eur J Immunol* 2004 Mar;**34**(3):623-630.

(98) Glinka Y, Prud'homme GJ. Neuropilin-1 is a receptor for transforming growth factor beta-1, activates its latent form, and promotes regulatory T cell activity. *J Leukoc Biol* 2008 Jul;**84**(1):302-310.

(99) Sarris M, Andersen KG, Randow F, Mayr L, Betz AG. Neuropilin-1 expression on regulatory T cells enhances their interactions with dendritic cells during antigen recognition. *Immunity* 2008 Mar;**28**(3):402-413.

(100) Kubach J, Lutter P, Bopp T, Stoll S, Becker C, Huter E, et al. Human CD4+CD25+ regulatory T cells: proteome analysis identifies galectin-10 as a novel marker essential for their anergy and suppressive function. *Blood* 2007 Sep 1;**110**(5):1550-1558.

(101) Wang J, Ioan-Facsinay A, van d, V, Huizinga TW, Toes RE. Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. *Eur J Immunol* 2007 Jan;**37**(1):129-138.

(102) Yamaguchi T, Hirota K, Nagahama K, Ohkawa K, Takahashi T, Nomura T, et al. Control of Immune Responses by Antigen-Specific Regulatory T Cells Expressing the Folate Receptor. *Immunity* 2007 Jul 27;**27**(1):145-159.

(103) Horwitz DA, Zheng SG, Wang J, Gray JD. Critical role of IL-2 and TGF-beta in generation, function and stabilization of Foxp3+CD4+ Treg. *Eur J Immunol* 2008 Apr;**38**(4):912-915.

(104) Huter EN, Punkosdy GA, Glass DD, Cheng LI, Ward JM, Shevach EM. TGF-beta-induced Foxp3+ regulatory T cells rescue scurfy mice. *Eur J Immunol* 2008 Jul;**38**(7):1814-1821.

(105) Shevach EM, Davidson TS, Huter EN, Dipaolo RA, Andersson J. Role of tgf-Beta in the induction of foxp3 expression and T regulatory cell function. *J Clin Immunol* 2008 Nov;**28**(6):640-646.

(106) Ochando JC, Yopp AC, Yang Y, Garin A, Li Y, Boros P, et al. Lymph node occupancy is required for the peripheral development of alloantigen-specific Foxp3+ regulatory T cells. *J Immunol* 2005 Jun 1;**174**(11):6993-7005.

(107) Li N, Workman CJ, Martin SM, Vignali DA. Biochemical analysis of the regulatory T cell protein lymphocyte activation gene-3 (LAG-3; CD223). *J Immunol* 2004 Dec 1;**173**(11):6806-6812.

(108) Liang B, Workman C, Lee J, Chew C, Dale BM, Colonna L, et al. Regulatory T cells inhibit dendritic cells by lymphocyte activation gene-3 engagement of MHC class II. *J Immunol* 2008 May 1;**180**(9):5916-5926.

(109) Yagi H, Nomura T, Nakamura K, Yamazaki S, Kitawaki T, Hori S, et al. Crucial role of FOXP3 in the development and function of human CD25+CD4+ regulatory T cells. *Int Immunol* 2004 Nov;**16**(11):1643-1656.

(110) Niedbala W, Wei XQ, Cai B, Hueber AJ, Leung BP, McInnes IB, et al. IL-35 is a novel cytokine with therapeutic effects against collagen-induced arthritis through the expansion of regulatory T cells and suppression of Th17 cells. *Eur J Immunol* 2007 Nov;**37**(11):3021-3029.

(111) Battaglia M, Stabilini A, Migliavacca B, Horejs-Hoeck J, Kaupper T, Roncarolo MG. Rapamycin promotes expansion of functional CD4+CD25+FOXP3+ regulatory T cells of both healthy subjects and type 1 diabetic patients. *J Immunol* 2006 Dec 15;**177**(12):8338-8347.

(112) Battaglia M, Stabilini A, Roncarolo MG. Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. *Blood* 2005 Jun 15;**105**(12):4743-4748.

(113) Knutson BA. Insights into the domain and repeat architecture of target of rapamycin. *J Struct Biol* 2010 May;**170**(2):354-363.

(114) Soulard A, Cohen A, Hall MN. TOR signaling in invertebrates. *Curr Opin Cell Biol* 2009 Dec;**21**(6):825-836.

(115) Otsubo Y, Yamamato M. TOR signaling in fission yeast. *Crit Rev Biochem Mol Biol* 2008 Jul;**43**(4):277-283.

(116) Qi H, Chen Y, Fu X, Lin CP, Zheng XF, Liu LF. TOR regulates cell death induced by telomere dysfunction in budding yeast. *PLoS One* 2008;**3**(10):e3520.

(117) Nakashima A, Sato T, Tamanoi F. Fission yeast TORC1 regulates phosphorylation of ribosomal S6 proteins in response to nutrients and its activity is inhibited by rapamycin. *J Cell Sci* 2010 Mar 1;**123**(Pt 5):777-786.

(118) Nakashima A, Maruki Y, Imamura Y, Kondo C, Kawamata T, Kawanishi I, et al. The yeast Tor signaling pathway is involved in G2/M transition via polo-kinase. *PLoS One* 2008;**3**(5):e2223.

(119) Danko I, Chapman V, Wolff JA. The frequency of revertants in *mdx* mouse genetic models for Duchenne muscular dystrophy. *Pediatr Res* 1992 Jul;**32**(1):128-131.

(120) Hoffman EP, Morgan JE, Watkins SC, Partridge TA. Somatic reversion/suppression of the mouse *mdx* phenotype in vivo. *J Neurol Sci* 1990 Oct;**99**(1):9-25.

(121) Sahashi K, Ibi T, Suoh H, Nakao N, Tashiro M, Marui K, et al. Immunostaining of dystrophin and utrophin in skeletal muscle of dystrophinopathies. *Intern Med* 1994 May;**33**(5):277-283.

(122) Thanh LT, Nguyen TM, Helliwell TR, Morris GE. Characterization of revertant muscle fibers in Duchenne muscular dystrophy, using exon-specific monoclonal antibodies against dystrophin. *Am J Hum Genet* 1995 Mar;**56**(3):725-731.

(123) Eghtesad S, Morel PA, Clemens PR. The companions: regulatory T cells and gene therapy. *Immunology* 2009 May;**127**(1):1-7.

(124) Sakaguchi S. Policing the regulators. *Nature Immunology* 2001 Apr 1;**2**(4):283-284.

(125) Cheng X, Dai H, Wan N, Moore Y, Vankayalapati R, Dai Z. Interaction of programmed death-1 and programmed death-1 ligand-1 contributes to testicular immune privilege. *Transplantation* 2009 Jun 27;**87**(12):1778-1786.

(126) Hagiwara H, Ohsawa Y, Asakura S, Murakami T, Teshima T, Sunada Y. Bone marrow transplantation improves outcome in a mouse model of congenital muscular dystrophy. *FEBS Lett* 2006 Aug 7;**580**(18):4463-4468.

(127) Camirand G, Stephan L, Rousseau J, Sackett MK, Caron NJ, Mills P, et al. Central tolerance to myogenic cell transplants does not include muscle neoantigens. *Transplantation* 2008 Jun 27;**85**(12):1791-1801.

(128) Feng SW, Lu XL, Liu ZS, Zhang YN, Liu TY, Li JL, et al. Dynamic distribution of bone marrow-derived mesenchymal stromal cells and change of pathology after infusing into *mdx* mice. *Cytotherapy* 2008;**10**(3):254-264.

(129) Quinlan JG, Lyden SP, Cambier DM, Johnson SR, Michaels SE, Denman DL. Radiation inhibition of *mdx* mouse muscle regeneration: dose and age factors. *Muscle Nerve* 1995 Feb;**18**(2):201-206.

(130) Chretien F, Dreyfus PA, Christov C, Caramelle P, Lagrange JL, Chazaud B, et al. In vivo fusion of circulating fluorescent cells with dystrophin-deficient myofibers results in extensive sarcoplasmic fluorescence expression but limited dystrophin sarcolemmal expression. *Am J Pathol* 2005 Jun;**166**(6):1741-1748.

(131) Mendell JR, Campbell K, Rodino-Klapac L, Sahenk Z, Shilling C, Lewis S, et al. Dystrophin immunity in Duchenne's muscular dystrophy. *N Engl J Med* 2010 Oct 7;**363**(15):1429-1437.

(132) Burleson RL, Marbarger PD, Jermanovich N, Brennan AM, Scruggs BF. A prospective study of methylprednisolone and prednisone as immunosuppressive agents in clinical renal transplantation. *Transplant Proc* 1981 Mar;**13**(1 Pt 1):339-343.

(133) Liu Y, Chen Y, Liu FQ, Lamb JR, Tam PK. Combined treatment with triptolide and rapamycin prolongs graft survival in a mouse model of cardiac transplantation. *Transpl Int* 2008 May;**21**(5):483-494.

(134) Vilquin JT, Asselin I, Guerette B, Kinoshita I, Roy R, Tremblay JP. Successful myoblast allotransplantation in *mdx* mice using rapamycin. *Transplantation* 1995 Feb 15;**59**(3):422-426.

(135) Thomson AW, Turnquist HR, Raimondi G. Immunoregulatory functions of mTOR inhibition. *Nat Rev Immunol* 2009 May;**9**(5):324-337.

(136) Libetta C, Sepe V, Zucchi M, Portalupi V, Meloni F, Rampino T, et al. The effect of sirolimus- or cyclosporine-based immunosuppression effects on T-cell subsets in vivo. *Kidney Int* 2007 Jul;**72**(1):114-120.

(137) Sarbassov DD, Ali SM, Sabatini DM. Growing roles for the mTOR pathway. *Curr Opin Cell Biol* 2005 Dec;**17**(6):596-603.

(138) Hedhli N, Pelat M, Depre C. Protein turnover in cardiac cell growth and survival. *Cardiovasc Res* 2005 Nov 1;**68**(2):186-196.

(139) Ikenoue T, Hong S, Inoki K. Monitoring mammalian target of rapamycin (mTOR) activity. *Methods Enzymol* 2009;**452**:165-180.

(140) Yang X, Yang C, Farberman A, Rideout TC, de Lange CF, France J, et al. The mammalian target of rapamycin-signaling pathway in regulating metabolism and growth. *J Anim Sci* 2008 Apr;**86**(14 Suppl):E36-E50.

(141) Zhou H, Huang S. The complexes of mammalian target of rapamycin. *Curr Protein Pept Sci* 2010 Sep 1;**11**(6):409-424.

(142) Bodine SC, Stitt TN, Gonzalez M, Kline WO, Stover GL, Bauerlein R, et al. Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. *Nat Cell Biol* 2001 Nov;**3**(11):1014-1019.

(143) Rommel C, Bodine SC, Clarke BA, Rossman R, Nunez L, Stitt TN, et al. Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. *Nat Cell Biol* 2001 Nov;**3**(11):1009-1013.

(144) Mouisel E, Vignaud A, Hourde C, Butler-Browne G, Ferry A. Muscle weakness and atrophy are associated with decreased regenerative capacity and changes in mTOR signaling in skeletal muscles of venerable (18-24-month-old) dystrophic *mdx* mice. *Muscle Nerve* 2010 Jun;**41**(6):809-818.

(145) Jhunjhunwala S, Raimondi G, Thomson AW, Little SR. Delivery of rapamycin to dendritic cells using degradable microparticles. *J Control Release* 2009 Feb 10;**133**(3):191-197.

(146) Strauss L, Whiteside TL, Knights A, Bergmann C, Knuth A, Zippelius A. Selective Survival of Naturally Occurring Human CD4+CD25+Foxp3+ Regulatory T Cells Cultured with Rapamycin. *J Immunol* 2007 Jan 1;**178**(1):320-329.

(147) Lange CM, Tran TY, Farnik H, Jungblut S, Born T, Wagner TO, et al. Increased frequency of regulatory T cells and selection of highly potent CD62L+ cells during treatment of human lung transplant recipients with rapamycin. *Transpl Int* 2010 Mar 1;**23**(3):266-276.

(148) Iannitti T, Capone S, Feder D, Palmieri B. Clinical use of immunosuppressants in duchenne muscular dystrophy. *J Clin Neuromuscul Dis* 2010 Sep;**12**(1):1-21.

(149) Charfi S, Krichen-Makni S, Yaich S, Makni H, Khabir A, Amouri A, et al. Successful treatment of post-renal transplant gastric and pulmonary Kaposi's sarcoma with conversion to rapamycin treatment. *Saudi J Kidney Dis Transpl* 2007 Nov;**18**(4):617-620.

(150) Iglesias J, Ortega J, Lopez JA, Sanchez L, Allende E, Asensio M, et al. Rapamycin-based rescue therapy after chronic rejection in a pediatric liver transplant patient. *Transpl Int* 2003 Oct;**16**(10):765-767.

(151) Kerkar N, Dugan C, Rumbo C, Morotti RA, Gondolesi G, Shneider BL, et al. Rapamycin successfully treats post-transplant autoimmune hepatitis. *Am J Transplant* 2005 May;**5**(5):1085-1089.

(152) Haxhinasto S, Mathis D, Benoist C. The AKT-mTOR axis regulates de novo differentiation of CD4+Foxp3+ cells. *J Exp Med* 2008 Feb 18;jem.

(153) Sauer S, Bruno L, Hertweck A, Finlay D, Leleu M, Spivakov M, et al. T cell receptor signaling controls Foxp3 expression via PI3K, Akt, and mTOR. *Proc Natl Acad Sci U S A* 2008 Jun 3;**105**(22):7797-7802.

(154) Stedman HH, Sweeney HL, Shrager JB, Maguire HC, Panettieri RA, Petrof B, et al. The *mdx* mouse diaphragm reproduces the degenerative changes of Duchenne muscular dystrophy. *Nature* 1991 Aug 8;**352**(6335):536-539.

(155) Drummond MJ, Fry CS, Glynn EL, Dreyer HC, Dhanani S, Timmerman KL, et al. Rapamycin administration in humans blocks the contraction-induced increase in skeletal muscle protein synthesis. *J Physiol* 2009 Apr 1;**587**(Pt 7):1535-1546.

(156) Risson V, Mazelin L, Roceri M, Sanchez H, Moncollin V, Corneloup C, et al. Muscle inactivation of mTOR causes metabolic and dystrophin defects leading to severe myopathy. *J Cell Biol* 2009 Dec 14;**187**(6):859-874.

(157) Wu B, Lu P, Benrashid E, Malik S, Ashar J, Doran TJ, et al. Dose-dependent restoration of dystrophin expression in cardiac muscle of dystrophic mice by systemically delivered morpholino. *Gene Ther* 2010 Jan;**17**(1):132-140.

(158) Esser K. Regulation of mTOR signaling in skeletal muscle hypertrophy. *J Musculoskelet Neuronal Interact* 2008 Oct;**8**(4):338-339.

(159) Ge Y, Wu AL, Warnes C, Liu J, Zhang C, Kawasome H, et al. mTOR regulates skeletal muscle regeneration in vivo through kinase-dependent and kinase-independent mechanisms. *Am J Physiol Cell Physiol* 2009 Dec;**297**(6):C1434-C1444.

(160) Pende M. mTOR, Akt, S6 kinases and the control of skeletal muscle growth. *Bull Cancer* 2006 May 1;**93**(5):E39-E43.

(161) Fluckey JD, Dupont-Versteegden EE, Knox M, Gaddy D, Tesch PA, Peterson CA. Insulin facilitation of muscle protein synthesis following resistance exercise in hindlimb-suspended rats is independent of a rapamycin-sensitive pathway. *Am J Physiol Endocrinol Metab* 2004 Dec;**287**(6):E1070-E1075.

(162) Fischer R, Turnquist HR, Taner T, Thomson AW. Use of rapamycin in the induction of tolerogenic dendritic cells. *Handb Exp Pharmacol* 2009;(188):215-232.

(163) Thomson AW. Immunosuppressive drugs and the induction of transplantation tolerance. *Transpl Immunol* 1994 Dec;**2**(4):263-270.

(164) Robinson J, Lai C, Martin A, Nye E, Tomlinson I, Silver A. Oral rapamycin reduces tumour burden and vascularization in Lkb1(+/-) mice. *J Pathol* 2009 Sep;**219**(1):35-40.

(165) Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, et al. Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. *Nature* 2009 Jul 16;**460**(7253):392-395.

(166) Hackstein H, Taner T, Zahorchak AF, Morelli AE, Logar AJ, Gessner A, et al. Rapamycin inhibits IL-4--induced dendritic cell maturation in vitro and dendritic cell mobilization and function in vivo. *Blood* 2003 Jun 1;**101**(11):4457-4463.

(167) Turnquist HR, Raimondi G, Zahorchak AF, Fischer RT, Wang Z, Thomson AW. Rapamycin-conditioned dendritic cells are poor stimulators of allogeneic CD4+ T cells, but enrich for antigen-specific Foxp3+ T regulatory cells and promote organ transplant tolerance. *J Immunol* 2007 Jun 1;**178**(11):7018-7031.

(168) Hartigan-O'Conner D, Kirk C, Crawford R, Mule J, Chamberlain J. Immune evasion by muscle-specific gene expression in dystrophic muscle. *Mol Ther* 2001 Dec 15;**4**(6):525-533.

(169) Wells DJ, Ferrer A, Wells KE. Immunological hurdles in the path to gene therapy for Duchenne muscular dystrophy. *Expert Rev Mol Med* 2002 Nov;**4**(23):1-23.

(170) Yang Y, Haecker S, Su Q, Wilson J. Immunology of gene therapy with adenoviral vectors in mouse skeletal muscle. *Human Molecular Genetics* 1996 Jun 15;**5**(11):1703-1712.

(171) Almond PS, Moss A, Nakhleh R, Melin M, Chen S, Salazar A, et al. Rapamycin in a porcine renal transplant model. *Transplant Proc* 1993 Feb;**25**(1 Pt 1):716.

(172) Battaglia M, Stabilini A, Draghici E, Gregori S, Mocchetti C, Bonifacio E, et al. Rapamycin and interleukin-10 treatment induces T regulatory type 1 cells that mediate antigen-specific transplantation tolerance. *Diabetes* 2006 Jan;**55**(1):40-49.

(173) Blazar BR, Taylor PA, Panoskaltsis-Mortari A, Vallera DA. Rapamycin inhibits the generation of graft-versus-host disease- and graft-versus-leukemia-causing T cells by interfering with the production of Th1 or Th1 cytotoxic cytokines. *J Immunol* 1998 Jun 1;**160**(11):5355-5365.

(174) Chen BJ, Morris RE, Chao NJ. Graft-versus-host disease prevention by rapamycin: cellular mechanisms. *Biol Blood Marrow Transplant* 2000;**6**(5A):529-536.

(175) Hayashi AA, Proud CG. The rapid activation of protein synthesis by growth hormone requires signaling through mTOR. *Am J Physiol Endocrinol Metab* 2007 Jun;**292**(6):E1647-E1655.

(176) Lawrence JC, Jr. mTOR-dependent control of skeletal muscle protein synthesis. *Int J Sport Nutr Exerc Metab* 2001 Dec;**11 Suppl**:S177-S185.

(177) Wang X, Proud CG. The mTOR pathway in the control of protein synthesis. *Physiology (Bethesda)* 2006 Oct;**21**:362-369.

(178) Eghtesad S, Zheng H, Nakai H, Epperly MW, Clemens PR. Effects of irradiating adult *mdx* mice before full-length dystrophin cDNA transfer on host anti-dystrophin immunity. *Gene Ther* 2010 Sep;**17**(9):1181-1190.

(179) Hickey EW, Hirshfield IN. Low-pH-induced effects on patterns of protein synthesis and on internal pH in Escherichia coli and Salmonella typhimurium. *Appl Environ Microbiol* 1990 Apr;**56**(4):1038-1045.

(180) Arechavala-Gomeza V, Kinali M, Feng L, Guglieri M, Edge G, Main M, et al. Revertant fibres and dystrophin traces in Duchenne muscular dystrophy: implication for clinical trials. *Neuromuscul Disord* 2010 May;**20**(5):295-301.

(181) Groh S, Zong H, Goddeeris MM, Lebakken CS, Venzke D, Pessin JE, et al. Sarcoglycan complex: implications for metabolic defects in muscular dystrophies. *J Biol Chem* 2009 Jul 17;**284**(29):19178-19182.

(182) Koenig M, Monaco AP, Kunkel LM. The complete sequence of dystrophin predicts a rod-shaped cytoskeletal protein. *Cell* 1988 Apr 22;**53**(2):219-228.

(183) Ferrer A, Foster H, Wells KE, Dickson G, Wells DJ. Long-term expression of full-length human dystrophin in transgenic *mdx* mice expressing internally deleted human dystrophins. *Gene Ther* 2004 Jun;**11**(11):884-893.

(184) Alajez NM, Schmielau J, Alter MD, Cascio M, Finn OJ. Therapeutic potential of a tumor-specific, MHC-unrestricted T-cell receptor expressed on effector cells of the innate and the adaptive immune system through bone marrow transduction and immune reconstitution. *Blood* 2005 Jun 15;**105**(12):4583-4589.

(185) BRIDGES JB, LOUTIT JF, MICKLEM HS. Transplantation immunity in the isologous mouse radiation chimaera. *Immunology* 1960 Jul;**3**:195-213.

(186) Cao ZA, Daniel D, Hanahan D. Sub-lethal radiation enhances anti-tumor immunotherapy in a transgenic mouse model of pancreatic cancer. *BMC Cancer* 2002 May 3;**2**:11.

(187) Goud SN. Effects of sublethal radiation on bone marrow cells: induction of apoptosis and inhibition of antibody formation. *Toxicology* 1999 Jul 15;**135**(2-3):69-76.

(188) Lugade AA, Sorensen EW, Gerber SA, Moran JP, Frelinger JG, Lord EM. Radiation-induced IFN-gamma production within the tumor microenvironment influences antitumor immunity. *J Immunol* 2008 Mar 1;**180**(5):3132-3139.

(189) Granchelli JA, Pollina C, Hudecki MS. Pre-clinical screening of drugs using the *mdx* mouse. *Neuromuscul Disord* 2000 Jun;**10**(4-5):235-239.

(190) Parreira SL, Resende MB, Della Corte PM, Marie SK, Carvalho MS, Reed UC. Quantification of muscle strength and motor ability in patients with Duchenne muscular dystrophy on steroid therapy. *Arq Neuropsiquiatr* 2007 Jun;**65**(2A):245-250.