Title

Effect of Cytotoxic T-Lymphocyte-Associated Protein 4 Immunoglobulin (CTLA-4Ig) Fusion Protein on Recruitment of Regulatory T Cells and Macrophages to Dystrophic Muscles in mdx Mouse Model of Duchenne Muscular Dystrophy

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A thesis submitted in partial satisfaction of the requirements for the degree

Master of Science in Physiological Science

by

Su-yin Kok

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ABSTRACT OF THE THESIS

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Su-yin Kok

Master of Science in Physiological Science

University of California, Los Angeles, 2017

Professor James G. Tidball, Chair

Duchenne muscular dystrophy (DMD) is a fatal X-linked disease characterized by chronic muscle degeneration. Because the immune system modulates the severity of DMD, it provides a potential therapeutic target for DMD. Previous investigations have shown that cytotoxic T-lymphocyte-associated protein 4 immunoglobulin (CTLA-4Ig) fusion protein inhibits T cell activation and reduces migration of macrophages. The objective of this study was to assess CTLA-4Ig as a potential DMD immunotherapy. Mdx mice, a mouse model of DMD, were administered CTLA-4Ig and euthanized at 4-week or 3-months old. At the peak of muscle inflammation, CTLA-4Ig-treated 4-week mice exhibited significantly reduced necrosis and reduced numbers of CD68+ M1 macrophages and CD163+ M2 macrophages in muscle and in

individual injured myofibers. At 3-months, we saw little treatment effects indicating that the influence of CTLA-4Ig is transient. Due to the reduction in necrosis and inflammatory cells in the dystrophic muscles, CTLA-4Ig is a promising immunotherapy for DMD.

The thesis of Su-yin Kok is approved.

Kenneth Dorshkind

Reggie Edgerton

James G. Tidball, Committee Chair

University of California, Los Angeles

2017

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a severe X-linked recessive disease that causes chronic muscle degeneration and premature death in male children. Mutations in the dystrophin gene can produce either the loss of the dystrophin protein or a dysfunctional dystrophin, resulting in structural abnormalities in the muscle tissue and weakening of muscle fiber strength and stability (Hoffman, et al. 1987). As the compromised muscle tissues undergo a cycle of inflammation and degeneration, followed by repair, healthy muscle tissues are eventually replaced by fibrous tissue and fat (Morrison, et al. 2000). The progressive deterioration of muscle contractility, beginning with the voluntary skeletal muscles and then affecting respiratory and cardiac muscles in later stages, causes patients with DMD to experience loss of mobility, cardiomyopathy, and ultimately death in their mid-twenties (Hilton, et al. 1993).

The discovery that DMD muscle contained elevated levels of lymphoid and myeloid cells (Arahata and Engel, 1984) suggested the immune response had a role in modulating the pathology of DMD. Dystrophic tissues in human patients and mdx mice, the murine model of DMD, contained elevated populations of T cells and myeloid cells (McDouall, et al. 2001; Cai, et al. 2000). These immune cells promoted the inflammation and pathophysiology of the muscle fibers (Spencer, et al. 2001; Villalta, et al. 2009). Thus, the reductions of T cell and myeloid cell populations in dystrophic tissues are attractive targets for therapeutic intervention.

Myeloid cells are integral to the pathophysiology of DMD as macrophages comprise the largest proportion of inflammatory cells in dystrophic tissues (Wehling, et al. 2001) and have been shown to promote cytolysis and regeneration of myofibers (Villalta, et al. 2009). Macrophages

have two phenotypes, M1 and M2. M1 macrophages are pro-inflammatory cells that are classically activated by IFN-γ, lipopolysaccharide (LPS) and TNF-α, and produce pro-inflammatory cytokines, such as TNF-α, IL-1, and IL-12 (Classen, et al. 2009). M1 macrophages contribute to a Th1 immune response, eventually resulting in cytotoxicity and tissue injury (Classen, et al. 2009). Conversely, M2 macrophages are anti-inflammatory cells that undergo alternative activation by IL-4, IL-13, and TGF-β, and produce anti-inflammatory cytokines, such as IL-10, TGF-β, and arginase (Classen, et al. 2009). M2 macrophages produce a Th2 immune response and contribute to tissue repair and wound healing (Classen, et al. 2009). Villalta et al. (2009) found elevated populations of M1 and M2 macrophages in 4-week old mdx mice, when the tissues were at peak inflammation and necrosis. The M1 macrophages were cytotoxic and induced lysis of damaged myofibers via an inducible nitric oxide synthase (iNOS)/nitric oxide (NO)-dependent pathway (Villalta, et al. 2009). M2 macrophages promoted tissue repair by inhibiting the cytotoxicity of M1 macrophages and reducing NO production via an arginase-1-dependent mechanism (Villalta, et al. 2009). In 3-month mdx tissues, when muscle pathology was reduced and myofibers were regenerated (Duddy, et al. (2015), the macrophage population had shifted to a primarily M2 phenotype (Villalta, et al 2009). Eosinophils are another cytolytic myeloid cell with elevated populations in dystrophic tissues. T cells can secrete chemokines, such as CCL3, that recruit eosinophils to inflammatory sites and once activated, eosinophils promote lysis of myofibers and fibrosis in mdx muscles (Cai, et al. 2000; Wehling-Henricks, et al. 2008). Their cytotoxic function is dependent on T cells as depletion of CD8+ T cells in dystrophic tissues diminished populations of eosinophils (Cai, et al. 2000). However, depletion of eosinophils produced increased numbers of CD8+ T cells, indicating a role for eosinophils in suppressing CTL numbers in dystrophic muscles (Wehling-Henricks, et al. 2008).

Lymphoid cells also play important roles in regulating the immune response to DMD, with CD4+ and CD8+ T cells promoting muscle pathology (Spencer, et al. 2001) and regulatory T cells alleviating inflammation and ameliorating the disease (Villalta, et al. 2014). CD4+ T cells are helper T cells (Th) that activate when they bind to a specific antigen-major histocompatibility complex II (MHC class II) (Lederman, et al. 1992). These cells bolster the immune response by releasing cytokines that activate macrophages and CD8+ cytotoxic T cells, and assist in the maturation of B cells (Lederman, et al. 1992). When CD8+ T cells (CTLs) bind to an antigen-major histocompatibility complex (MHC) class I, they activate and become cytotoxic, effectively neutralizing damaged or infected cells (Berg and Forman, 2006). Mdx tissues contained increased levels of CD4+ and CD8+ T cells, which were specifically activated in the dystrophic muscles as the lymph nodes did not show an accompanying increase in activated T cells (Spencer, et al. 2001). Depletion of these effector T cells produced a significant reduction in mdx muscle pathology, suggesting these T cells play a key role in the progression and severity of DMD (Spencer, et al. 2001). Regulatory T cells (Tregs) counteract the effects of CD4+ and CD8+ T cells by suppressing the immune response to maintain immune homeostasis, prevent autoimmunity, and terminate the cytolytic activity of T cells when their function is completed (Betteli, et al. 2006). Tregs also play a regulatory role in dystrophic muscle. For example, depletion of Tregs increased pathology in mdx mice and experimental manipulations that increased Tregs showed reduced inflammation and pathology (Villalta, et al. 2014). Tregs could also reduce DMD pathology by suppressing effector T cell populations. Tregs suppress effector T cells by blocking a costimulatory signal necessary for the activation of T cells. Effector T cells are activated in a two-signal process. The first signal occurs when the T cell receptor (TCR) binds its specific antigen-MHC class I or class II presented by an antigen-presenting cell (APC).

The second non-specific, costimulatory signal occurs when the CD28 receptor on the T cell binds the B7 ligands, B7-1 and B7-2 (CD80/86) on the APC, initiating activation of the T cell (Greenfield, et al. 1998). Tregs constitutively express cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), a surface protein that blocks the costimulation pathway by preferentially binding B7 proteins on APCs and preventing the binding of the CD28 receptor (Deppong et al. 2013). Since the T cell does not receive the costimulatory signal, the T cell becomes anergic (Sucher et al. 2012).

By manipulating the populations of myeloid and T cells at the height of inflammation in dystrophic muscles, the immune response could be influenced to favor an anti-inflammatory, regenerative state and to minimize pro-inflammatory factors. The fusion protein, CTLA-4Ig, which consists of the extracellular domains of CTLA-4 fused to immunoglobulin G1 (IgG1), suppresses the T cell alloreactive response *in vitro* (Takahashi et al, 2014) and increases the percentage of Tregs, identified as $CD4^{+}CD25^{high}Foxp3^{+}$, both *in vitro* and *in vivo* (Razmara, et al. 2008). *In vitro* experiments suggest CTLA-4Ig does not increase the population of CD4+CD25+Foxp3+ cells by expanding the population of naturally occurring Tregs, but rather via the conversion of CD4+CD25- T cells (Razmara, et al. 2008). This expansion of Foxp3+ Tregs appears to be dependent on APCs, which were T-cell depleted splenocytes for this study, as irradiated APCs were not able to induce proliferation of Foxp3+ Tregs (Razmara, et al. 2008). Forkhead box p3 (Foxp3) is a transcription factor that functions as a master regulator in the development and function of Tregs, and is expressed in all CD4+ Tregs (Zhang and Zhao, 2007).

However, CTLA-4Ig can also hinder the migratory capability of monocytes in human patients

afflicted with rheumatoid arthritis (Bonelli, et al. 2013). Patients treated with CTLA-4Ig increased levels of monocytes in their blood, indicating the monocytes did not exit the blood vessel and travel to the site of inflammation. CTLA-4Ig-treated patients also showed a significant decrease in the expression of adhesion molecules, CD15, CD50, CD54, CD58, CD62E, and CD106 (VCAM-1). Thus, CTLA-4Ig negatively affected leukocyte extravasation. Another study showed that CTLA-4Ig downregulated the expression of CD80/86 on primary cultures of macrophages from patients with rheumatoid arthritis (Cutolo and Nadler, 2013). The downregulation of B7.1-2 (CD80/86) on macrophages, which are APCs, would obstruct the costimulatory signal and prevent activation of T cells. In addition, this compromise in the function of macrophages as APCs may negatively affect the ability of CTLA-4Ig to expand the Treg population, as suggested by the findings from the Razmara, et al. (2008).

Since gene therapies have not yet yielded a successful cure for DMD, we are exploring immunotherapies to mitigate the pathology of DMD by reducing T cell and myeloid cell populations in the dystrophic tissues. The objective of this study is to determine the effects of CTLA-4Ig on the recruitment of Tregs and macrophages to dystrophic muscles in 4-week and 3-month old mdx mice, and observe its effects on the overall disease phenotype. We hypothesize that administration of CTLA-4Ig will improve the physiology of dystrophic mdx tissues by decreasing the recruitment of M1 and M2 macrophages to the dystrophic muscles and increasing the population of Tregs.

MATERIALS AND METHODS

Animals

Mdx mice were obtained from our breeding colony housed in a pathogen-free vivarium at UCLA. The facility utilized a 12-hour light and a 12-hour dark cycle. Original breeding pairs were purchased from The Jackson Laboratory. All animal experiments were conducted in accordance with guidelines issued by the UCLA Animal Research Committee.

Mdx mice were analyzed at 4 weeks of age, which represents early, acute peak of inflammation and muscle damage, and at 3 months of age, when inflammation and pathology have attenuated and muscles are undergoing regeneration. The 4-week group consisted of 9 mice (control group consisted of 5 mice – 3 females and 2 males; treatment group consisted of 4 mice – 2 females and 2 males). The 3-month group consisted of 9 mice (control group consisted of 3 mice – 1 female and 2 males; treatment group consisted of 6 mice – 1 female and 5 males). Following treatment, 4-week mice were euthanized at 4 weeks of age and 3-month mice at 3 months of age using isoflurane. Prior to dissection, the body mass (g) of each mouse was recorded. Both hindlimb muscles were carefully dissected to minimize any artificial stretching of the muscles and their weights (mg) were recorded. Right hindlimb muscles were embedded in Tissue Tek O.C.T, flash-frozen in liquid-nitrogen cooled isopentane, and stored at -80^{o}C in vials containing isopentane. Left hindlimb muscles were stored in cryovials and flash frozen in liquid nitrogen, before being stored in a cryogenic storage tank containing liquid nitrogen.

CTLA-4Ig Treatment

Mice received intraperitoneal injections of CTLA-4Ig or human IgG isotype control (25 μg/g

body weight) (BioXCell) at 23, 25, and 27 days of age. Mice were sacrificed for tissue collection at day 28 (4-week old group) or at day 90 (3-month old group). CTLA-4Ig and human IgG isotype control were diluted in sterile phosphate-buffered saline (PBS) to a final volume of 100 µl. Prior to each injection, mice were weighed and dosage was adjusted according to weight.

Recruitment and Infiltration of Macrophages in Myofibers

Quadriceps from the right hindlimbs were sectioned at a thickness of 10 µm. Sectioned tissues were air dried for 30 minutes. Tissues were fixed in cold acetone for 10 minutes, air dried again for 10 minutes, washed in PBS for 5 minutes, quenched with 0.3% hydrogen peroxide in PBS for 10 minutes and washed in PBS. Tissues were blocked in a buffer containing 0.2% gelatin, 0.05% Tween-20, and 3% BSA for 30 minutes and washed in PBS. Tissues were incubated with primary antibody for 3 hours at room temperature (RT) (CD68, 1:100 [Serotec] and CD163, 1:100 [Santa Cruz]) or overnight at 4^0C (CD206, 1:50 [Serotec]), washed three times in PBS, incubated with host-specific secondary antibody of biotinylated IgG for 30 minutes (1:200, Vector Laboratories), washed three times in PBS, and incubated with Avidin-D HRP for 30 minutes (1:1000, Vector Laboratories). Tissues were visualized with AEC substrate kit (Vector Laboratories). After sealing with VectaMount (Vector Laboratories), stained tissues were stored at -20^{o}C.

Macrophage recruitment was quantified as the number of positive cells outside the myofibers and within the endomysium or perimysium. Macrophage infiltration was quantified as the number of positive cells inside the myofibers. Macrophage density was quantified as the total number of positive cells per volume of tissue (mm^3). Positive cells inside blood vessels or in the

epimysium were not included in the counts.

Recruitment of Tregs in Myofibers

Quadriceps from the right hindlimbs were sectioned at a thickness of 10 μm. Sectioned tissues were air dried for 30 minutes. Tissues were fixed in 2% paraformaldehyde in PBS for 5 minutes, washed in PBS for 5 minutes, quenched with 0.3% hydrogen peroxide in PBS for 10 minutes and washed in PBS. Tissues were blocked in a buffer containing 0.2% gelatin, 0.05% Tween-20, and 3% BSA for 1 hour. Tissues were then incubated with Foxp3 antibody (1:50, Abcam) for 3 hours at RT, washed three times in PBS, incubated with secondary antibody of biotinylated goat anti-rabbit IgG for 30 minutes (1:200, Vector Laboratories), washed three times in PBS, and incubated with Avidin-D HRP for 30 minutes (1:1000, Vector Laboratories). Tissues were visualized with AEC substrate kit (Vector Laboratories). After sealing with VectaMount (Vector Laboratories), stained tissues were stored at -20^{o}C.

Treg recruitment was quantified as the number of positive cells outside the myofibers. Treg density was quantified as the total number of positive cells per volume of tissue (mm^3). Positive cells inside blood vessels or in the epimysium were not included in the counts.

Muscle Fiber Morphology – Cross-sectional area, regeneration, and necrosis

Quadriceps from the right hindlimbs were sectioned at a thickness of 10 μm and stained with filtered hematoxylin (Gill's formula, Vector Laboratories). The myofiber cross-sectional area (CSA) of each muscle was quantified as the average CSA (mm^2) of 500 myofibers per quadriceps. CSA was calculated with Bioquant Image Analysis software at a magnification of

100x. Only CSAs of circled myofibers with a shape value greater than 0.50 were included in the data. The variances of the myofiber CSAs of each quadriceps were also calculated. Regeneration of muscle fibers was quantified as the number of centrally nucleated muscle fibers per total fibers in each quadriceps. Regenerating myofibers display central nucleation whereas healthy, mature myofibers have their nuclei in the periphery (Folker and Baylies, 2013).

Necrosis was determined via staining for IgG within muscle fibers. IgG is normally found outside healthy myofibers but crosses the compromised membrane of necrotic fibers into the interior of the necrotic myofiber. Quadriceps from the right hindlimbs were sectioned at a thickness of 10 μm. Sectioned tissues were air dried for 30 minutes. Tissues were fixed in cold acetone for 10 minutes, air dried again for 10 minutes, washed in PBS for 5 minutes, blocked in 1% gelatin in PBS, and washed in PBS. Tissues were incubated with IgG antibody for overnight at 4^0C (IgG DyLight 594, 1:100 [Vector Laboratories]). Tissues were washed three times in PBS and mounted in ProLong Gold antifade mountant. Necrosis was quantified as the number of muscle fibers with interior staining of IgG per total fibers in quadriceps.

RNA Extraction

For each mouse, 80 - 100 mg of the left hamstring was excised and homogenized in Trizol (Life Technologies) using a Sorvall omni-mixer. Tissues were homogenized at least three times for 30-second periods interspersed with 20-second intervals with tissues placed on ice. Homogenized samples were spun at 12,000 g for 10 minutes at 4^oC to remove insoluble material and then incubated at RT for 5 minutes to allow complete dissociation of nucleoprotein complex. Chloroform (20% of original Trizol volume) was added to lyse the cell membranes. Samples

were spun at 12,000 g for 15 minutes at 4°C to separate samples into three phases: a clear, aqueous, top layer containing the RNA, a middle layer containing protein, and a red, bottom layer containing organic, phenol-chloroform. The top layer with RNA was carefully extracted, with special care to not disturb or uptake the middle layer. RNA samples were incubated with isopropanol (50% of original Trizol volume) at RT for 10 minutes and then spun at 12,000 g for 10 minutes at 4°C. The resulting small white pellet of RNA was resuspended in 75% ethanol in autoclaved diethyl pyrocarbonate (DEPC) water and spun at 7,500 g for 5 minutes at 4°C. After removing the supernatant, the white pellet was air dried on ice for 5 minutes and then resuspended in RNAse-free water. The RNA was purified using RNeasy Mini Kit (Qiagen), per manufacturer's instructions. RNA quantity was assessed via a Beckman DU730 spectrophotometer and all samples achieved an OD260:OD280 ratio greater than 1.8. RNA quality was visualized on a 1.2% agarose gel.

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

A total volume of 10 µl of 2 µg of RNA and autoclaved water was added to a Master Mix containing10 mM dNTPs and Oligo$(dT)_{12\text{-}18}$ primer (Invitrogen). The samples were heated at 65°C for 5 minutes to denature the secondary structure of RNA, then incubated on ice for 5 minutes to allow the primer to anneal to the RNA strands. A second Master Mix containing SuperScript II (Invitrogen) was added. Samples were heated at 42°C for 50 minutes to bind the DNA polymerase and then heated at 70°C for 15 minutes to inactivate the enzyme. The final cDNA sample is diluted 1:1 in autoclaved water and stored at -20°C.

qPCR was performed with each well containing a total of 25 µl of sample – 2 µl of cDNA and 23

μl Master Mix containing autoclaved water, Sybr Green nucleic acid stain (Biorad), and the appropriate forward and reverse primers. Housekeeping genes were RPNS1 and HPRT1 for 4-week tissues, and RPNS1 and RPL13A for 3-month tissues. Experimental genes were CD4, CD8, CD68, CD163, CD206, Foxp3, IFN- γ, IL-4, IL-10, IP-10, MCP-1, Pax7, Siglec F, and TNF-α.

Statistical Analysis

Statistical analyses were calculated using the non-parametric Mann-Whitney U test. The p-value was set at $p < 0.5$.

RESULTS

Myeloid Cells

Recruitment and Infiltration of M1 and M2 Macrophages

The 4-week mice treated with CTLA-4Ig showed significant decreases both in the numbers of CD68+ M1 macrophages in the inflamed tissues (Fig. 1a) and the infiltration of these CD68+ M1 macrophages into the muscle fibers (Fig. 1b). However, levels of CD68 mRNA showed a slight, non-significant increase (Fig. 1c). This could be attributed to the presence of CD68+ cells in the muscle homogenates used for RNA isolation that were not included in the immunohistochemistry (IHC) data, either because they were located in the epimysium or inside blood vessels because CTLA-4Ig may have reduced their ability to extravasate. The 3-month mice showed no significant differences between the two groups. The density of CD68+ macrophages in the tissues of 3-month mice was lower than in the 4-week mice, but it is interesting to note that a greater proportion of CD68+ macrophages invaded the 3-month myofibers compared to the 4-week group.

Analyses of CD163+ M2 macrophages in 4-week mice showed significant decreases in the recruitment of (Fig. 1d,j) and invasion of these M2 macrophages into the muscle fibers (Fig. 1e, k). There was no difference in levels of CD163 mRNA in the 4-week mice (Fig. 1f). CD163+ M2 macrophages were recruited to the muscles of the 3-month mice (Fig. 1d) but none of these macrophages infiltrated the muscle fibers (Fig. 1e). qPCR analysis showed that the 3-month CTLA-4Ig-treated mice exhibited a significant increase in CD163 mRNA (Fig. 1f).

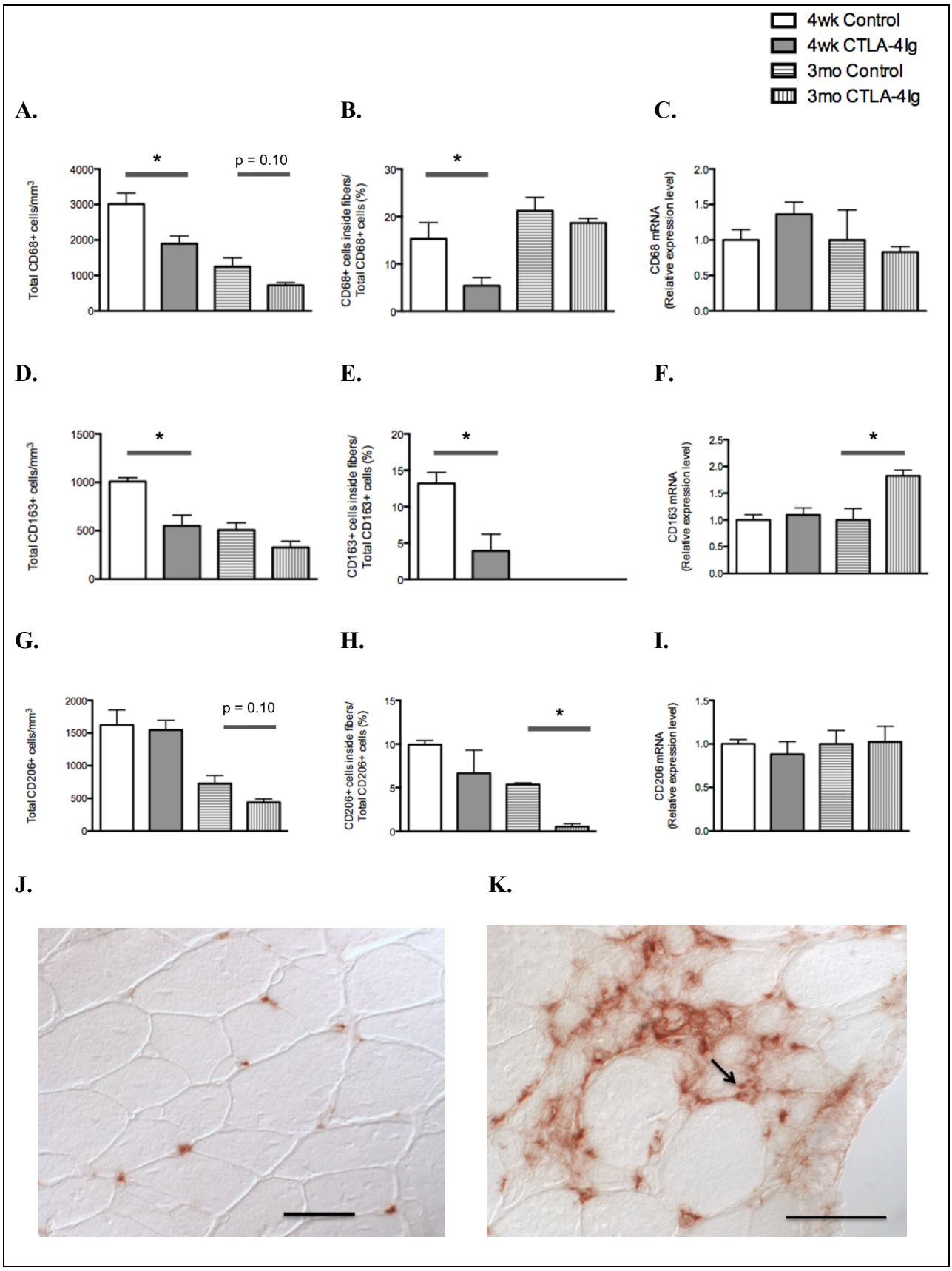
4wk Control
4wk CTLA-4Ig
3mo Control
3mo CTLA-4Ig
A.
B.
C.
Total CD68+ cells/mm3
CD68+ cells inside fibers/ Total CD68+ cells (%)
CD68 mRNA (Relative expression level)
*
p = 0.10
D.
E.
F.
Total CD163+ cells/mm3
CD163+ cells inside fibers/ Total CD163+ cells (%)
CD163 mRNA (Relative expression level)
G.
H.
I.
Total CD206+ cells/mm3
CD206+ cells inside fibers/ Total CD206+ cells (%)
CD206 mRNA (Relative expression level)
J.
K.

Figure 1. CTLA-4Ig significantly diminished the recruitment of CD68+ M1 macrophages and CD163+ M2 macrophages to the dystrophic tissues of 4-week mice and their infiltration into the muscle fibers. **A.** IHC analysis of CD68+ cells/mm^3 of quadriceps from both 4-week and 3-month mdx mice treated with CTLA-4Ig or human isotype control. CTLA-4Ig significantly decreased the density of CD68+ cells/mm^3 in 4-week mdx mice. **B.** IHC analysis of infiltration (%) of CD68+ cells inside muscle fiber/total CD68+ cells in quadriceps of both 4-week and 3-month mdx mice treated with CTLA-4Ig or human isotype control. CTLA-4Ig significantly decreased the infiltration of CD68+ cells into muscle fibers in 4-week mdx mice. **C.** qPCR analysis of CD68 mRNA in hamstrings of 4-week and 3-month mdx mice treated with CTLA-4Ig or human isotype control. CTLA-4Ig did not have a significant effect on either 4-week or 3-month mdx mice. **D.** IHC analysis of CD163+ cells/mm^3 from quadriceps of both 4-week and 3-month mdx mice treated with CTLA-4Ig or human isotype control. CTLA-4Ig significantly decreased the density of CD163+ cells/mm^3 in 4-week mdx mice. **E.** IHC analysis of infiltration (%) of CD163+ cells inside muscle fiber/total CD163+ cells in quadriceps of both 4-week and 3-month mdx mice treated with CTLA-4Ig or human isotype control. CTLA-4Ig significantly decreased the infiltration of CD163+ cells into muscle fibers in 4-week mdx mice. **F.** qPCR analysis of CD163 mRNA in hamstrings of 4-week and 3-month mdx mice treated with CTLA-4Ig or human isotype control. CTLA-4Ig significantly increased CD163 mRNA in 3-month mdx mice. **G.** IHC analysis of CD206+ cells/mm^3 from quadriceps of both 4-week and 3-month mdx mice treated with CTLA-4Ig or human isotype control. CTLA-4Ig did not significantly affect density of CD206+ cells/mm^3 in 4-week and 3-month mdx mice. **H.** IHC analysis of infiltration (%) of CD206+ cells inside muscle fiber/total CD206+ cells in quadriceps of both 4-week and 3-month mdx mice treated with CTLA-4Ig or human isotype control. CTLA-4Ig significantly decreased the infiltration of CD206+ cells into muscle fibers in 3-month mdx mice. **I.** qPCR analysis of CD206 mRNA in hamstrings of 4-week and 3-month mdx mice treated with CTLA-4Ig or human isotype control. CTLA-4Ig did not have a significant effect on either 4-week or 3-month mdx mice. **J.** Representative image of CD68+ cell outside a muscle fiber in 3-month mdx quadriceps. Scale bar = 50 μm. **K.** Representative image of CD68+ cell inside a necrotic fiber in 4-week mdx quadriceps. Arrow indicates CD68+ cell. Scale bar = 50 μm. *, $p < 0.05$.

Neither the 4-week or 3-month mice showed significant differences between the treatment groups in the recruitment of CD206+ M2 macrophages to the tissues (Fig. 1g) or in mRNA levels (Fig. 1i). However, the 3-month mice treated with CTLA-4Ig showed a significant decrease of infiltration of CD206+ M2 macrophages into the muscle fibers, while the 4-week CTLA-4Ig-treated mice showed a slight but non-significant decrease (Fig. 1h).

Chemokine and Cytokine Environment

Because previous investigators showed that CTLA-4Ig could reduce expression of chemokines that attract inflammatory cells into diseased tissue (Nakayama, et al. 2005), we tested whether similar effects were observed in CTLA-4Ig-treated mice. Nakayama, et al. (2005) showed that, 7 days post-treatment, CTLA-4Ig reduced the mRNA expression of monocyte chemoattractant protein-1 (MCP-1) and IFN-γ-induced protein-10 (IP-10) in the livers of mice with fulminant hepatitis. MCP-1 (or CCL2) is a chemokine that recruits monocytes, dendritic cells, and memory T cells to sites of tissue injury or inflammation (Deshmane, 2009). IP-10 (or CXCL10) is another key chemokine secreted by monocytes, endothelial cells, and fibroblasts and is involved in the chemoattraction of macrophages and T cells (Dufour, et al. 2002). Thus, these two chemokines play an important role in regulating the migration of macrophages and may explain how CTLA-4Ig reduced the recruitment of CD68+ M1 macrophage and CD163+ M2 macrophages to dystrophic 4-week mdx tissues.

However, both our 4-week and 3-month mdx hamstrings did not show a significant difference in expression levels of MCP-1 mRNA (Fig. 2a) or IP-10 mRNA (Fig. 2b).

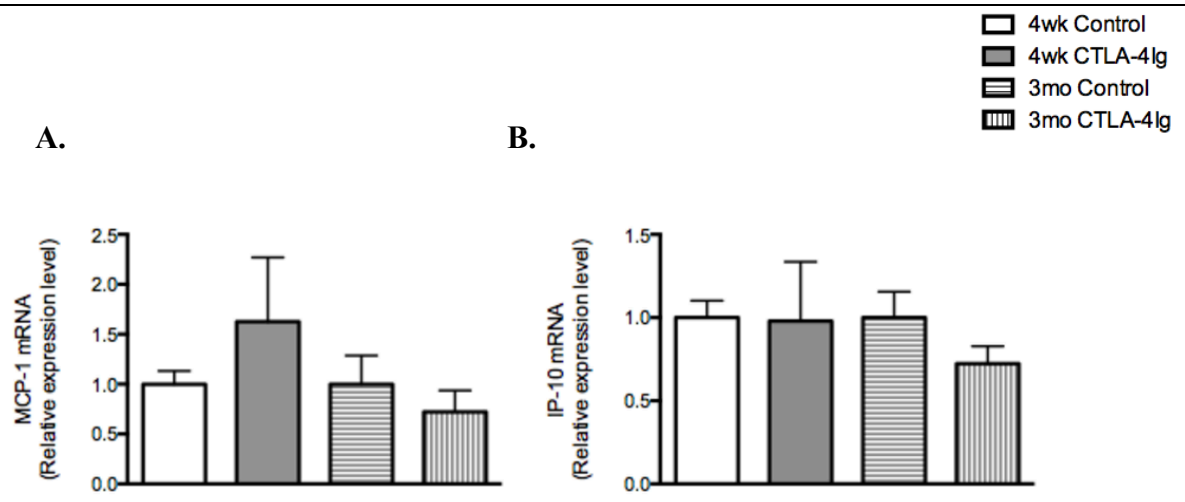

Figure 2. CTLA-4Ig did not significantly affect mRNA expression levels of chemokines that regulate migration of macrophages, MCP-1 and IP-10. **A.** qPCR analysis of MCP-1 mRNA in hamstrings of 4-week and 3-month mdx mice treated with CTLA-4Ig or human isotype control. CTLA-4Ig did not have a significant effect on either 4-week or 3-month mdx mice. **B.** qPCR analysis of IP-10 mRNA in hamstrings of 4-week and 3-month mdx mice treated with CTLA-4Ig or human isotype control. CTLA-4Ig did not have a significant effect on either 4-week or 3-month mdx mice. *, $p < 0.05$.

Because Cutolo and Nadler (2013) showed that CTLA-4Ig significantly reduced the production of pro-inflammatory cytokines by the synovial macrophages, we tested whether CTLA-4Ig reduced the cytokines necessary to induce macrophages to undergo classical (M1) or alternative (M2) activation. IFN-γ and TNF-α are pro-inflammatory cytokines that induce classical activation of macrophages to the M1 phenotype.

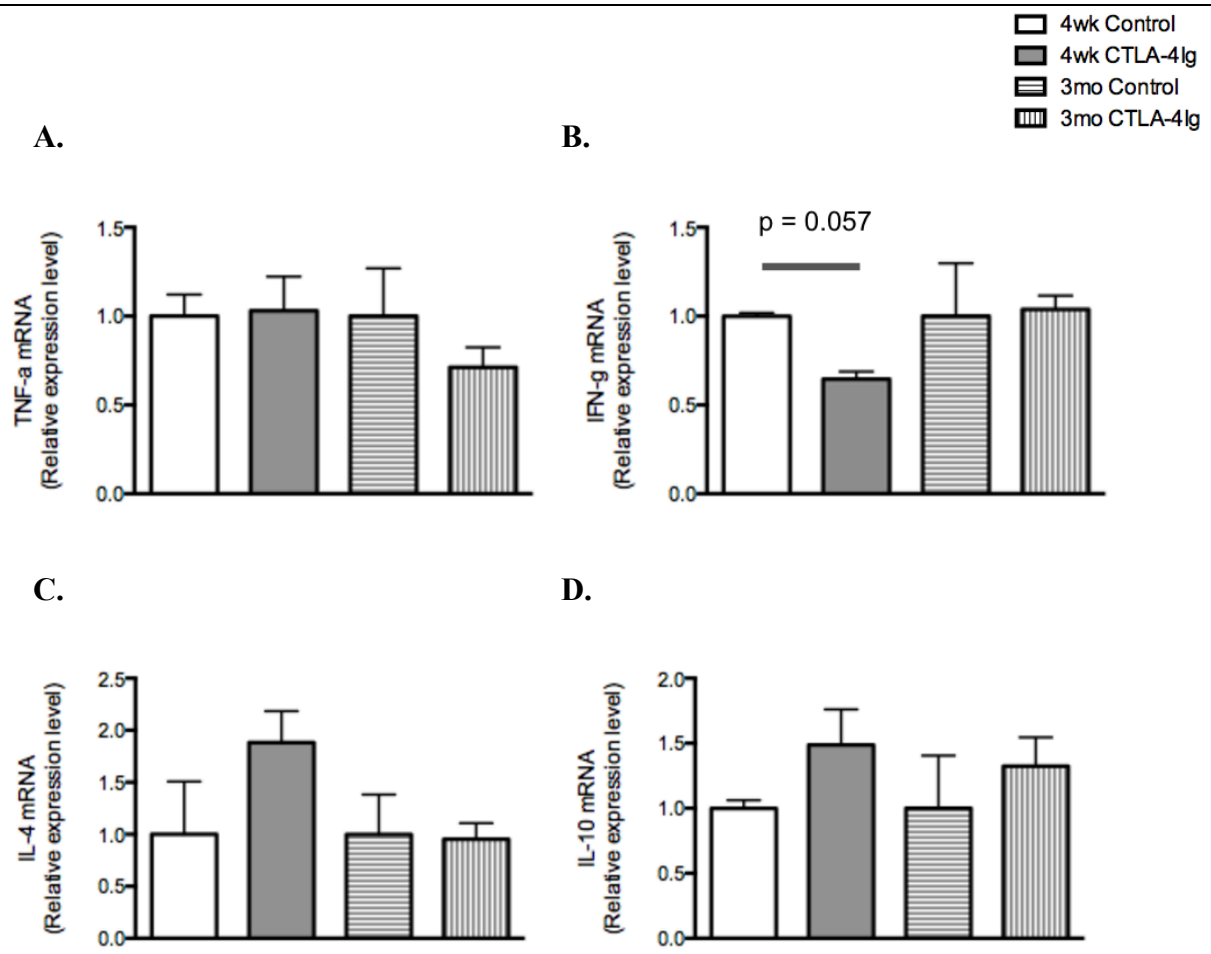

Figure 3. CTLA-4Ig did not significantly affect mRNA expression levels of cytokines that induce M1 macrophage phenotype, IFN-γ and TNF-α, or induce M2 macrophage, IL-4 and IL-10. **A.** qPCR analysis of TNF-α mRNA in hamstrings of 4-week and 3-month mdx mice treated with CTLA-4Ig or human isotype control. CTLA-4Ig did not have a significant effect on either 4-week or 3-month mdx mice. **B.** qPCR analysis of IFN-γ mRNA in hamstrings of 4-week and 3-month mdx mice treated with CTLA-4Ig or human isotype control. CTLA-4Ig did not have a significant effect on either 4-week or 3-month mdx mice, but CTLA-4Ig-treated mice had a strong trending decrease of IFN-γ mRNA in 4-week mdx mice ($p = 0.057$). **C.** qPCR analysis of IL-4 mRNA in hamstrings of 4-week and 3-month mdx mice treated with CTLA-4Ig or human isotype control. CTLA-4Ig did not have a significant effect on either 4-week or 3-month mdx mice. **D.** qPCR analysis of IL-10 mRNA in hamstrings of 4-week and 3-month mdx mice treated with CTLA-4Ig or human isotype control. CTLA-4Ig did not have a significant effect on either 4-week or 3-month mdx mice. *, $p < 0.05$.

Though there were no significant changes between treatment groups in TNF-α mRNA expression for both 4-week and 3-month mdx mice (Fig. 3a), CTLA-4Ig produced a strong trending decrease in IFN-γ mRNA expression in the 4-week mdx mice ($p = 0.057$) (Fig. 3b). CTLA-4Ig did not produce a significant effect in the 3-month mdx mice. IL-4 and IL-10 are anti-inflammatory cytokines that induce alternative activation of M2 macrophages. Though there was a general trend of increase of IL-4 (Fig. 3c) and IL-10 mRNA expression (Fig. 3d), especially in the 4-week mice, no differences were statistically significant between CTLA-4Ig-treated or control mice at either age compared.

Eosinophils

CTLA-4Ig did not produce a significant effect in Siglec F mRNA, a marker for eosinophils, in 4-week and 3-month mdx mice (Fig. 4).

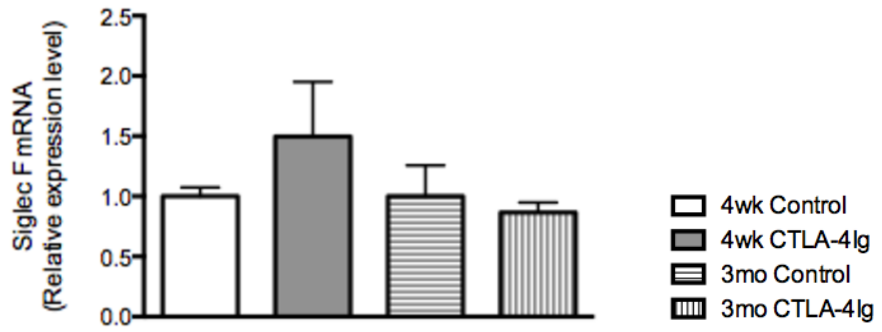

Figure 4. CTLA-4Ig did not affect mRNA expression of eosinophils. **A.** qPCR analysis of Siglec F mRNA in hamstrings of 4-week and 3-month mdx mice treated with CTLA-4Ig or human isotype control. CTLA-4Ig did not have a significant effect on either 4-week or 3-month mdx mice. *, $p < 0.05$.

Lymphoid Cells

Effector T cells

Because CTLA-4Ig can inhibit activation of T cells , we assayed mRNA levels of CD4 and CD8. Though CLTA-4Ig did not significantly affect CD4 mRNA in either 4-week or 3-month mice (Fig. 5a), there was a significant decrease of CD8 mRNA in 4-week mice (Fig. 5b).

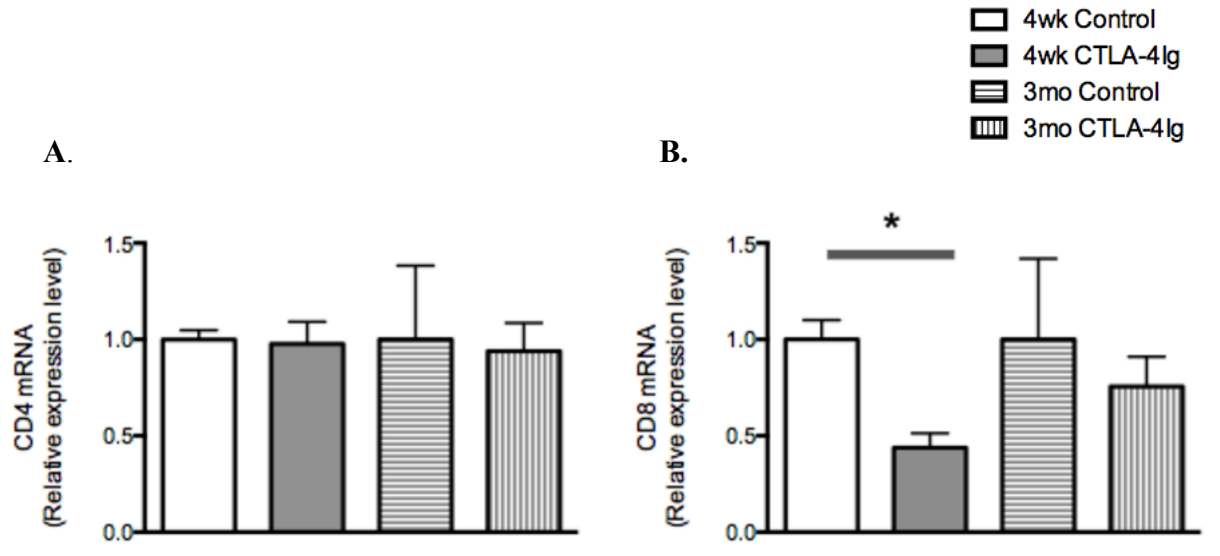

Figure 5. CTLA-4Ig did not affect mRNA expression of CD4+ T cells and eosinophils, but it significantly decreased mRNA of CD8+ T cells in 4-week mdx mice. **A.** qPCR analysis of CD4 mRNA in hamstrings of 4-week and 3-month mdx mice treated with CTLA-4Ig or human isotype control. CTLA-4Ig did not have a significant effect on either 4-week or 3-month mdx mice. **B.** qPCR analysis of CD8 mRNA in hamstrings of 4-week and 3-month mdx mice treated with CTLA-4Ig or human isotype control. CTLA-4Ig significantly decreased CD8 mRNA in 4-week mdx mice. *, $p < 0.05$.

Regulatory T cells

Because Razmara, et al. (2008) showed CTLA-4Ig increased the percentage of Foxp3+ Tregs *in*

vivo, we expected to see a similar occurrence, especially in the inflamed muscles of the 4-week mice. Surprisingly, the 4-week mdx mice treated with CTLA-4Ig exhibited a trending decrease in numbers of Foxp3+ cells and a significant decrease in Foxp3 mRNA (Fig. 6). The 3-month CTLA-4Ig-treated mice exhibited a significant decrease in numbers of Foxp3+ cells, though there was a trending increase in levels of Foxp3 mRNA (Fig. 6).

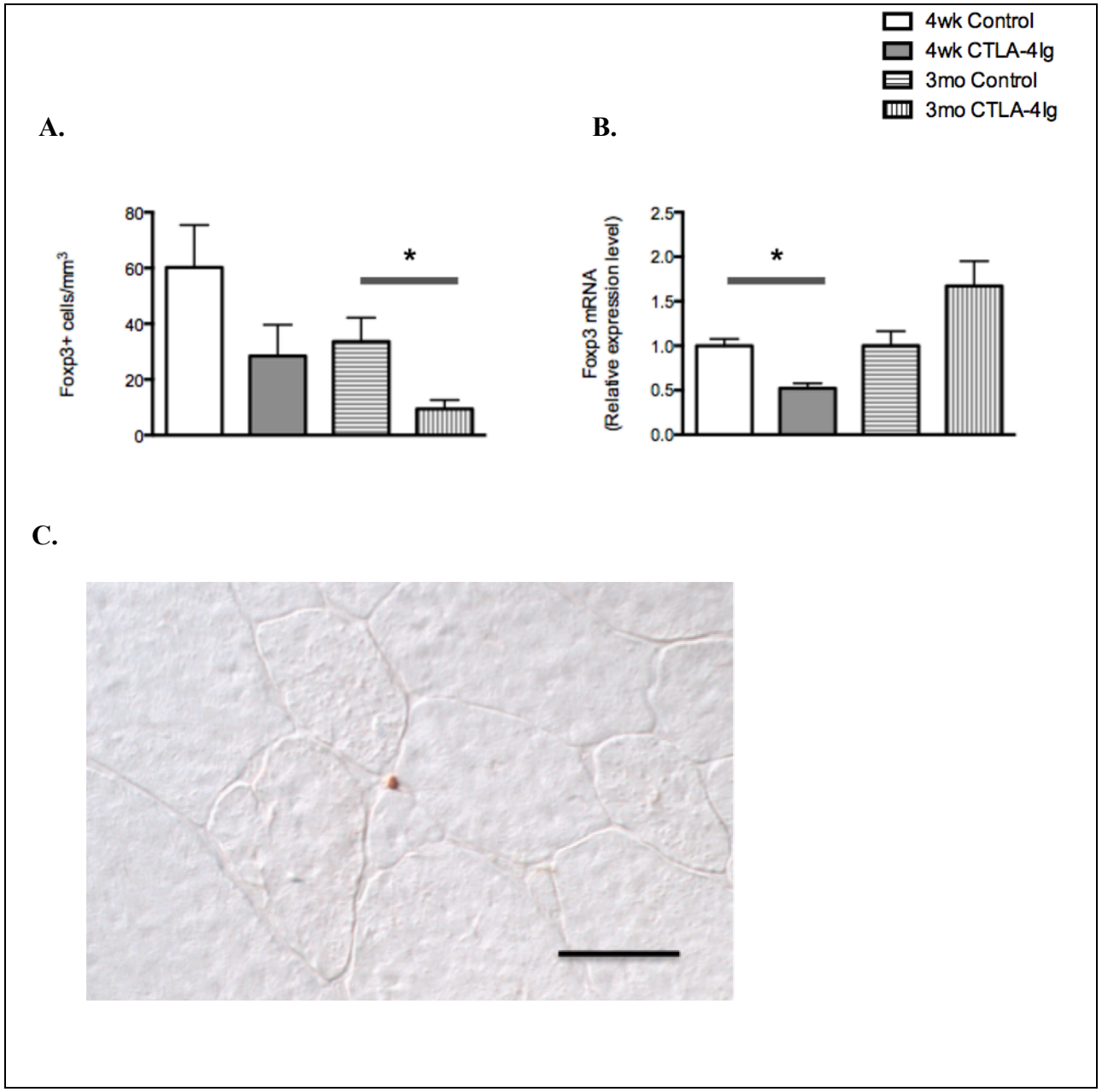

Figure 6. CTLA-4Ig reduced the number of Foxp3+ cells in both 4-week and 3-month mdx mice, and reduced Foxp3 mRNA in 4-week mdx mice. **A.** IHC analysis of Foxp3+ cells/mm^3 from quadriceps of both 4-week and 3-month mdx mice treated with CTLA-4Ig or human isotype control. CTLA-4Ig significantly decreased the density of Foxp3+ cells/mm^3 in 3-month mdx mice **B.** qPCR analysis of Foxp3 mRNA in hamstrings of 4-week and 3-month mdx mice treated with CTLA-4Ig or human isotype control. CTLA-4Ig significantly decreased Foxp3 mRNA in hamstrings of 4-week mdx mice. **C.** Representative image of Foxp3+ cell outside a muscle fiber in 3-month mdx quadriceps. Scale bar = 50 μm. *, $p < 0.05$.

Muscle Morphology and Health

Necrosis

A necrosis analysis revealed that 4-week mdx mice treated with CTLA-4Ig experienced a significant reduction in necrosis (Fig. 7a-b).

Myofiber CSA

As there is a strong positive correlation between the CSA of a muscle and the maximum force produced by the muscle, CSA is used as indicator of muscle growth and regeneration (Liu, et al. 1985). Variance was calculated to determine the distribution of the CSAs of each myofiber. CTLA-4Ig did not produce a significant effect in either 4-week or 3-month mdx mice for myofiber CSAs and their variances (Fig. 7c-d).

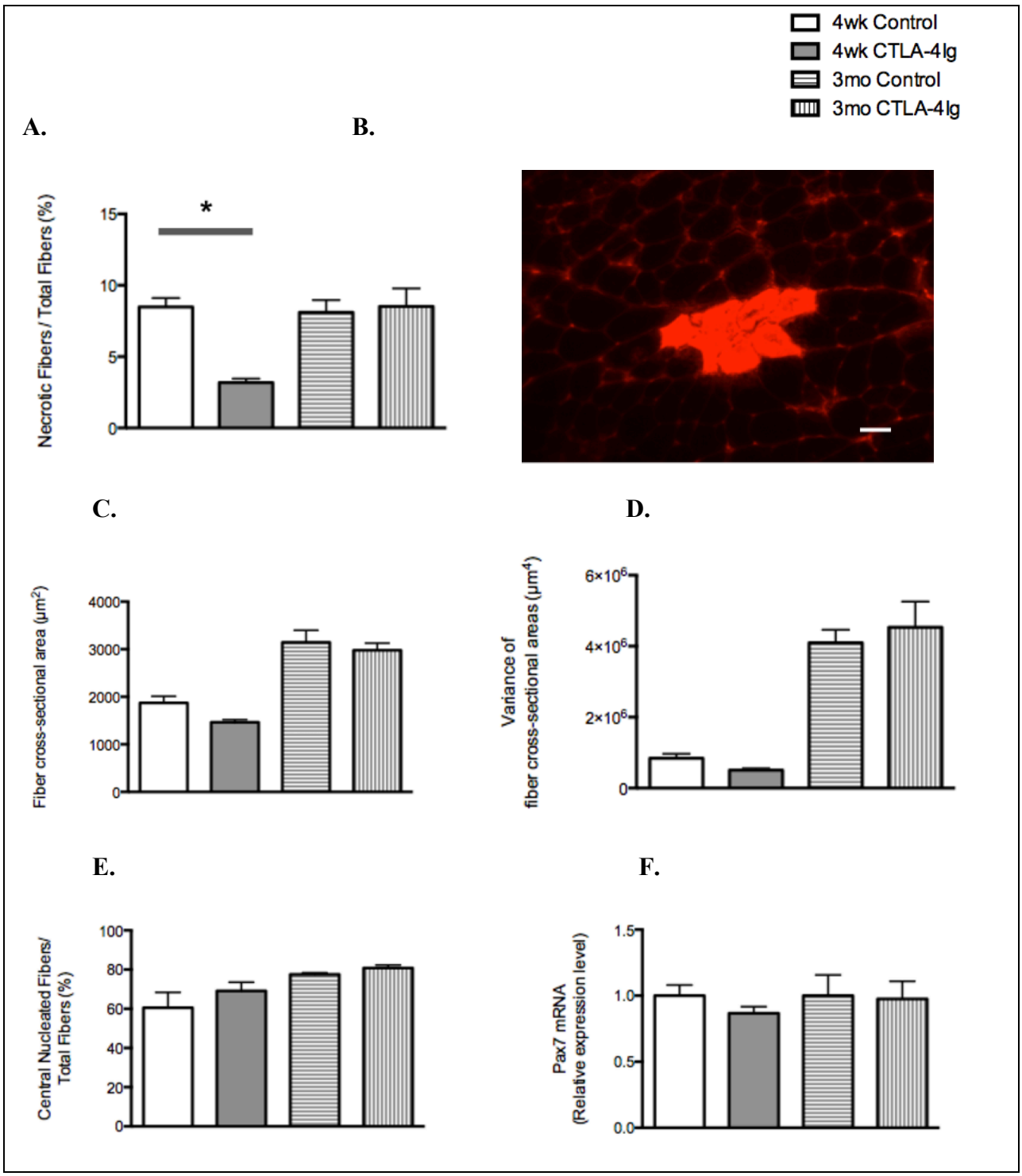
4wk Control
4wk CTLA-4Ig
3mo Control
3mo CTLA-4Ig
A.
B.
Necrotic Fibers / Total Fibers (%)
15
10
5
0
*
C.
D.
Fiber cross-sectional area (μm²)
4000
3000
2000
1000
0
Variance of fiber cross-sectional areas (μm⁴)
6×10⁶
4×10⁶
2×10⁶
0
E.
F.
Central Nucleated Fibers/ Total Fibers (%)
100
80
60
40
20
0
Pax7 mRNA (Relative expression level)
1.5
1.0
0.5
0.0

Figure 7. CTLA-4Ig significantly decreased necrosis in 4-week mdx mice but did not have an effect on other indicators of muscle morphology, myofiber CSA and regeneration. **A.** IHC analysis of proportion (%) of necrotic IgG+ fibers/total fibers of quadriceps of both 4-week and 3-month mdx mice treated with CTLA-4Ig or human isotype control. CTLA-4Ig significantly decreased muscle necrosis in 4-week mdx mice. **B.** Representative image of IgG staining inside a necrotic fiber in 3-month mdx quadriceps. Scale bar = 50 μm. **C-D.** Myofiber CSAs (μm^2) were calculated for 500 myofibers/quadriceps. Mean CSA (μm^2) and variance (μm^4) were calculated for each quadriceps of 4-week and 3-month mdx mice treated with CTLA-4Ig or human isotype control. CTLA-4Ig did not have a significant effect on either 4-week or 3-month mdx mice. **E.** Muscle regeneration calculated as percentage of centrally nucleated fibers/total fibers of quadriceps of 4-week and 3-month mdx mice treated with CTLA-4Ig or human isotype control. CTLA-4Ig did not have a significant effect on either 4-week or 3-month mdx mice. **E.** qPCR analysis of Pax7 mRNA in hamstrings of 4-week and 3-month mdx mice treated with CTLA-4Ig or human isotype control. CTLA-4Ig did not have a significant effect on either 4-week or 3-month mdx mice. *, $p < 0.05$.

Regeneration

Other indicators of muscle health and stability are the capacity for regeneration and repair of tissue after injury, and the ability to replace necrotic myofibers by forming new muscles (Carlson and Faulkner, 1983). Satellite cells are instrumental in the regeneration process. This population of muscle stem cells is usually quiescent but proliferates in response to injury (Morgan and Partridge, 2003). These cells can self-renew and produce more satellite cells, provide additional myonuclei to muscle fibers, or form new muscle fibers by undergoing myogenesis and differentiating into myoblasts (Yin, et al. 2013). Regenerating myofibers display central nucleation whereas healthy, mature myofibers have their nuclei in the periphery (Folker and Baylies, 2013). CTLA-4Ig did not produce a significant effect in either 4-week or 3-month mdx mice for proportion of regenerating, centrally nucleated myofibers (Fig. 7e), and mRNA levels of Pax7 mRNA, a marker for satellite cells (Fig. 7f).

Discussion

In this study, we showed that CTLA-4Ig modulated the inflammatory immune response in mdx mice and reduced the muscle pathophysiology. Most notably, CTLA-4Ig-treated mice exhibited significant reduction of necrosis and influx of inflammatory cells in the dystrophic muscles at 4-weeks, when tissues were at peak levels of inflammation and necrosis. In 4-week mice, CTLA-4Ig significantly reduced the numbers of CD68+ M1 macrophages, both within the tissue and invading the myofibers, and the expression of CD8 mRNA. Thus, the reduced necrosis could be due to the diminished presence of pro-inflammatory M1 macrophages and the potential reduction of CD8+ cytotoxic T cells, both of which are responsible for initiating the inflammatory response in dystrophic muscles (Villalta, et al. 2009; Spencer, et al. 1997).

However, the therapeutic effects of CTLA-4Ig appear to be transient because 3-month mdx mice did not display similar results. In 3-month mice, there were no significant differences in necrosis, CD68+ M1 macrophages, and CD8 mRNA levels between the two treatment groups, suggesting the drug has been eliminated from the physiological system. The 4-week mice were administered CTLA-4Ig less than a week prior to euthanization, allowing time for the drug to have an effect but not yet metabolize out of their systems. Additional tests need to be conducted to determine the frequency of dosage to maintain its efficacy and the toxicity of the drug.

Surprisingly, we saw a CTLA-4Ig-mediated decrease in Foxp3+ Tregs. 4-week CTLA-4Ig-treated mdx mice exhibited a trending decrease in numbers of Foxp3+ cells and a significant decrease in Foxp3 mRNA expression. This effect was still present at 3-months, with the CTLA-4Ig-treated mice displaying a significant decrease in numbers of Foxp3+ cells. One explanation

for these results is that Tregs were not recruited to the dystrophic muscles since CTLA-4Ig suppressed inflammation in 4-week mice, precluding the need for Tregs to localize to the tissue and downregulate the immune response. Another explanation for the decrease in Foxp3+ cells and mRNA is that when T cell receptors are ligated, as with CTLA-4Ig in this study, naïve T cells can either be induced into Th17 cells or Tregs, depending on the cytokine environment. If the environment contains TGF-β, $Foxp3^+$ Tregs are induced (Fu et al. 2004). If the environment contains TGF-β and IL-6 or IL-21, Th17 cells are induced (Qin et al. 2009). In a study conducted by Vogel, et al. (2015), CTLA-4Ig treatment increased disease in the mouse model for multiple sclerosis, decreased the suppressive function of Tregs, and increased expression of IL-17 in the lymph nodes. To further clarify this scenario, IHC and/or qPCR analyses can be conducted to determine if the cytokine environment contains IL-6 and/or IL-21, and IL-17, which is secreted by Th17 cells. Lastly, Razmara, et al. (2008) found that proliferation of the Tregs is dependent on APCs. Because CTLA-4Ig downregulated B7 ligands on macrophages (Cutolo and Nadler, 2013), it may have compromised the function of the macrophages and macrophages were unable to support expansion of Tregs.

The effects of CTLA-4Ig on CD68+ M1 and CD163+ M2 macrophages support the findings of Bonelli, et al. (2013) that CTLA-4Ig reduced the ability of monocytes to migrate out of the blood vessels into sites of inflammation. CTLA-4Ig-treated 4-week mice exhibited significantly reduced recruitment of CD68+ M1 and CD163+ M2 macrophages to the hindlimb muscles and also significantly hindered the ability of these macrophages to invade the dystrophic muscle fibers. Yu, et al. (2013) also showed that CTLA-4Ig reduced the migratory capacity of podocytes, renal cells that expressed B7.1 (CD80) when stimulated with LPS and displayed

immune-like properties. The next step would be to investigate how CTLA-4Ig reduces the migratory capacity of macrophages. Perhaps it involves the downregulation of B7.1-2 ligands (CD80/86) by CTLA-4Ig (Cutolo and Nadler, 2013), which may play a necessary role in the ability of these cells to migrate.

Though it was expected that the density of CD68+ cells in 3-month mdx mice would be less than in 4-week mdx mice, it is interesting to note that the proportion of CD68+ cells invading the muscle fibers was higher in the 3-month mdx mice compared to the 4-week mdx mice. Perhaps this indicates that the CD68+ M1 macrophages that travel to the dystrophic tissues at 3-months are more active or aggressive in clearing damaged tissue. CTLA-4Ig did not affect the mRNA levels of CD68 but it did produce a significant increase in the CD163 mRNA of 3-month mdx mice, possibly indicating an increase in CD163+ cells in the muscles at a later stage to further aid the regeneration process.

No significant changes by CTLA-4Ig were seen in expression of CD206+ cells and CD206 mRNA, except for a significant decrease of CD206+ M2 macrophages invading the tissues of 3-month mdx mice. This could suggest that differences between the CD163+ and CD206+ M2 macrophages largely protected the CD206+ M2 macrophages from CTLA-4Ig-mediated loss of migration, allowing these M2 macrophages to extravasate to the site of inflammation.

Though CTLA-4Ig decreased the recruitment of M1 pro-inflammatory macrophages and CD8 mRNA in 4-week mdx tissues, it had no effect on eosinophils, which are another pro-inflammatory myeloid cell with elevated populations in dystrophic tissues (Cai, et al. 2000).

Both 4-week and 3-month mice exhibited no differences in expression of Siglec F mRNA, a marker for eosinophils, between the two treatment groups. This could have been due to potential CTLA-4Ig-mediated reductions in CD8 T cells, and perhaps CD4 T cells. Because T cells are the primary recruiters of eosinophils to dystrophic tissues (Rand, et al. 1991), a reduction in T cell populations would result in no recruitment of eosinophils and thus, no changes in numbers of eosinophils in the tissues. An IHC analysis should be conducted to confirm the findings from the qPCR analysis.

To explain the CTLA-4Ig-mediated decrease of M1 and M2 macrophages in the 4-week dystrophic tissues, we assayed the mRNA expression levels of MCP-1 and IP-10, two chemokines responsible for the chemoattraction of macrophages to inflamed areas, as well as mRNA of IFN-γ, TNF-α, IL-4 and IL-10, cytokines that induce classical (M1) activation or alternative (M2) activation of macrophages. Unlike the results of Nakayama, et al. (2005), which showed CTLA-4Ig significantly decreasing both MCP-1 and IP-10 mRNA 7 days after treatment, neither the 4-week nor 3-month groups showed any significant differences in MCP-1 and IP-10 mRNA levels between the treatment groups. However, the relationship between mRNA and protein expression can be poorly correlated so an IHC analysis of MCP-1 and IP-10 in the tissues would be beneficial. Russell, et al. (1996) showed that CTLA-4Ig markedly reduced expression of MCP-1+ cells in cardiac tissues, 73 days post treatment, reduced expression of IFN-γ expression in cells, and fewer macrophages. These findings align with our results on macrophage density and IFN- γ mRNA expression. Bonelli, et al. (2013) also showed that CTLA-4Ig significantly reduced expression of CD106 (VCAM-1), an adhesion molecule on endothelial cells that facilitates leukocyte extravasation. 4-week and 3-month mdx tissues could

be tested for the presence of VCAM-1 and its ligand, α4β1, to determine if a decrease in these adhesion molecules are responsible for the inability of macrophages to extravasate.

qPCR analysis revealed that CTLA-4Ig did not have an effect on TNF-α mRNA, a pro-inflammatory cytokine, but a strong trending decrease was seen in IFN-γ mRNA ($p = 0.057$) in 4-week mice. This reduction in IFN-γ could reflect the reduced recruitment of CD68+ M1 macrophages seen in 4-week mdx tissues, since IFN-γ is a key cytokine that activates macrophages to the M1 phenotype. This reduction in IFN-γ mRNA could also be caused by a reduction in CD8+ T cells, which produce the pro-inflammatory cytokine. CTLA-4Ig did not have an effect on IL-4 and IL-10 mRNA levels in both 4-week and 3-month mdx mice. These cytokines are responsible for skewing macrophages to an M2 phenotype. It should also be noted that Tregs are a key producer of IL-10 (Villalta, 2014), so perhaps the combined secretion of IL-10 by Tregs and M2 macrophages helped to increase IL-10 mRNA levels. Thus, it appears that CTLA-4Ig does not reduce recruitment of M1 and M2 macrophages by preventing activation of these macrophages.

Though we saw a significant decrease in Tregs, there were no significant changes in CD4 mRNA expression between the treatment groups in both 4-week and 3-month mdx mice. Naturally occurring Tregs comprise approximately 5-10% of the total CD4+ T cell population (Seddiki, et al. 2006) so the decrease in Tregs may not have been enough to affect CD4 mRNA levels, which encompassed the total population of CD4+ T cells. However, CTLA-4Ig did significantly decrease mRNA expression of CD8 in 4-week mdx mice. Because CTLA-4Ig inhibits activation of T cells by blocking the CD28-B7 costimulatory signal (Deppong, et al. 2013), a decrease in

CD8 mRNA is expected but perhaps this indicates that CTLA-4Ig preferentially targets CD8+ T cells for inhibition over CD4+ T cells. Alternatively, the decrease of both Foxp3 and CD8 mRNA could reflect a decrease in the CD8+Foxp3+ Tregs population, which has been shown to also be effective in suppressing effector T cells (Churlaud et al. 2015). However, since it appears that the subset of CD8+Foxp3+ Tregs make up a small proportion of Tregs (Churlaud, et al. 2015), it is more likely that the decrease in CD8 mRNA is due to decreased expression of CD8+ T cells in the tissues. An IHC analysis should be conducted to ascertain that this translates to a marked reduction in CD8+ T cell population and activity in the tissues.

In both 4-week and 3-month mdx mice, CTLA-4Ig did not have a significant effect on the myofiber CSA or regenerative capacity, as measured by presence of centrally nucleated fibers and mRNA expression of satellite cells. As expected, the myofiber CSA in 3-month mdx mice were larger than in 4-week mdx, suggesting that CTLA-4Ig did not impede the ability of the myofibers to reach the more stable, healthier stage of the disease that is normally achieved at 3 months. Because CTLA-4Ig did not affect regeneration, the positive effects seen in reduction of necrosis is due to a reduction of inflammatory cells and not to an increase in the regenerative capacity of the muscles.

In summary, CTLA-4Ig may be a novel immunotherapy to reduce the pathology in patients with DMD. At the height of the disease in mdx mice, CTLA-4Ig produced markedly lower levels of necrosis and populations of inflammatory cells. We believe this warrants further investigation of CTLA-4Ig to determine its long term effects on the disease phenotype.

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