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Prednisone and CTLA4-Ig combined treatment reduces muscle pathology in mdx muscular dystrophy

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Haley Nicole Gamboa

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ABSTRACT OF THE THESIS

Prednisone and CTLA4-Ig combined treatment reduces muscle pathology in *mdx* muscular dystrophy

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Master of Science in Physiological Science

University of California, Los Angeles, 2021

Professor James G. Tidball, Chair

Although there is no cure for Duchenne muscular dystrophy (DMD), patients are commonly prescribed corticosteroids, typically prednisone, to slow disease progression and dampen the immune response. Prednisone promotes transcription of anti-inflammatory genes and reduces transcription of pro-inflammatory genes, leading to downregulation of prostaglandins that recruit immune cells. Because the immune system modulates the severity of pathology in DMD and in *mdx* mice (a genetic model for DMD), immunotherapy may also be beneficial. Cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) is a negative regulator of the immune system that inhibits activation of cytotoxic T-cells. CTLA4-Ig is a fusion protein that blocks the activation and invasion of macrophages and cytotoxic T-cells in other inflammatory and autoimmune diseases, which suggests that it may also positively affect the pathology of muscular dystrophy. Our lab previously demonstrated that CTLA4-Ig treatment alone is effective in reducing many aspects of *mdx* pathology. The objective of this study is to determine if the beneficial effects of

utilizing CTLA4-Ig are affected by co-administration with prednisone in an *mdx* mouse model. At the peak of muscular pathology, we found that the combined treatment of prednisone and CTLA4-Ig reduced inflammation, necrosis, and fibrosis in 4-week *mdx* quadriceps. Because of these promising outcomes, the combination of immunotherapy and corticosteroids may serve as a potential treatment for patients with DMD.

The thesis of Haley Nicole Gamboa is approved.

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2021

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Introduction

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder characterized by progressive skeletal muscle degeneration and weakness due to the absence of the protein dystrophin (Brockdorff et al. 1987). Dystrophin binds to the actin cytoskeleton and connects the cytoskeleton the dystrophin-associated glycoprotein complex, which serves a mechanical function for muscle fibers and is imperative in maintaining muscle integrity (Hoffman et al. 1987). However, the absence of dystrophin in DMD causes the muscle fiber membrane to be extremely weak, leading to influx of immune cells and eventually fiber death (Ervasti et al. 1990).

Although the immune response is necessary for normal repair during acute muscle damage, DMD pathology involves chronic muscle damage, which is exacerbated by the invasion of immune cells (McDouall et al. 1990). Levels of myeloid and lymphoid cells are heightened in patients with DMD (Arahata et al. 1984). Because these inflammatory cells promote DMD pathology (Villalta et al. 2009), they may be a promising therapeutic target.

Myeloid cells, specifically macrophages, are the most numerous inflammatory cells in dystrophic tissue (Wehling et al. 2001). Macrophages exist over a broad, continuous spectrum of phenotypes, spanning from the polarized M1 to M2 phenotypes (Mills et al. 2012). M1 macrophages are pro-inflammatory macrophages that are activated by cytokines such as IFNγ and produce cytokines like TNF-α (Classen et al. 2009). M1 macrophages are cytolytic and a main source of injury of dystrophic muscle (Villalta et a. 2009). Depletion of M1 macrophages in the *mdx* mouse model of DMD reduces myofiber damage (Wehling et al. 2001). Alternatively, M2

macrophages are anti-inflammatory and promote myogenesis. M2 macrophages are activated by cytokines such as IL-4 and produce cytokines such as IL-10 (Classen et al. 2009). The release of IL-10 de-activates M1 macrophages in muscle tissue (Villalta et al. 2011). In 4-week *mdx* muscles, cytolytic M1 macrophages comprise a larger proportion of the macrophage population (Villalta et al. 2011). After the acute stage of pathology, the macrophage phenotype switches to a predominantly M2, beginning the regenerative phase of DMD (Villalta et al. 2011). However, M2 macrophages also contribute to fibrogenesis in DMD by secreting a substrate for collagen synthesis, which leads to accumulation of connective tissue (Wehling et al. 2010).

Lymphoid cells also contribute to the pathophysiology of DMD even though they represent a much smaller percentage of the inflammatory cell population (Spencer et al. 1997). The main lymphoid cells present in DMD include CD4+ helper T-cells and CD8+ cytotoxic T-cells (Spencer et al. 1997, 2001). CD8+ cytotoxic T-cells can directly cause apoptosis of infected cells by recognizing antigens on major histocompatibility complex I (MHC-1) on the surface of antigen-presenting cells (Zhang et al. 2005). CD8+ cytotoxic T-cells lead to necrosis and apoptosis of muscle fibers via perforin-mediated cytotoxicity (Spencer et al. 1997). Depletions of CD8+ cytotoxic T-cells reduces necrosis (Spencer et al. 1997). CD4+ helper T-cells can contribute to M1 macrophage activation via release of IFNγ (Ma et al. 2003) and depletion of CD4+ helper T-cells also reduces muscle fiber damage (Spencer et al. 2001).

Another subset of CD4+ cells are FOXP3+ regulatory T-cells, which play a large role in immune regulation (Villalta et al. 2014). Regulatory T-cells are negative regulators of the immune system that maintain homeostasis of activated T-cells and can reduce pathology in *mdx* tissue (Villalta et

al. 2014). Depletion of FOXP3+ regulatory T-cells leads to increased inflammatory cell presence thereby increasing fiber damage (Villalta et al. 2014).

Thus, modulation of the immune system could improve DMD pathology. Prednisone is the most commonly prescribed treatment for DMD, but has broad effects on the immune system. Prednisone reduces transcription of pro-inflammatory genes and increases transcription of anti-inflammatory genes, leading to a variety of systemic effects (Barnes et al. 2006). Our lab demonstrated that prednisone reduced the number of macrophages and CD8+ T-cells in 4-week *mdx* quadriceps (Wehling-Henricks et al. 2004), thereby ameliorating dystrophic pathology. Because CD8+ T-cells and macrophages can cause necrosis, prednisone reduces percentage of fiber injury in an *mdx* model (Wehling-Henricks et al. 2004). Although prednisone reduces inflammation and necrosis in patients with DMD, it does so in a non-specific manner and has a variety of other systemic effects (Quattrocelli et al. 2017). If other therapies could directly modulate the activation of inflammatory cells, perhaps the pathophysiology of DMD could be improved.

Our lab has explored the possibility of using immunotherapy that could directly affect the interactions between immune cells as a therapeutic approach for muscular dystrophy. Co-stimulatory molecules are very important in the activation of inflammatory cells. For instance, T-cell activation first occurs by MHC-1 presentation of an antigen on antigen-presenting cells to the T-cell receptor located on T-cells (Herrero-Beaumont et al. 2012). The second portion of co-stimulation occurs by the T-cell surface molecule CD28 binding to CD80/86 on antigen-presenting cells (Herrero-Beaumont et al. 2012). The T-cell surface receptor cytotoxic T

lymphocyte-associated antigen-4 (CTLA4) is a negative regulator of the immune system that is presented on T-cells only after co-stimulation of CD28:CD80/86 (Gardner et al. 2014). CTLA4-Ig, or abatacept, is a recombinant fusion protein that consists of CTLA4 bound to the Fc portion of an IgG (Najafian et al. 2000). Blockade of stimulation of CD80/86 by CTLA4-Ig inhibits the activation of T cells and disrupts the immune memory process (Gardner et al. 2014). CTLA4-Ig is also a stronger ligand for CD80/86 than is CD28 (Herrero-Beaumont et al. 2012). Since CD28 and CTLA4 are imperative regulators of T cell function, they pose as potential therapeutic targets for treatment of immune-related diseases. In comparison to prednisone, CTLA4-Ig is a more specific immune regulator.

CTLA4-Ig is already an FDA approved treatment for adults and children as young as two years of age. Additionally, CTLA4-Ig decreases activation of the immune system in many animal models of autoimmune diseases (Gardner et al. 2014). CTLA4-Ig generates an intrinsic inhibitory signal that switches off T cell activation, immune cell proliferation, and inflammatory cytokine production (Gardner et al. 2014). By decreasing collagen I expression (Cutolo et al. 2013), CTLA4-Ig also reduces fibrosis in induced-dermal fibrosis in mice (Ponsoye et al. 2016) and rheumatoid arthritis in humans (Cutolo et al. 2009). Other studies show that CTLA4-Ig may act through a regulatory T-cell mediated mechanism to further block the activation and reproduction of effector T-cells (Deppong et al. 2013). CTLA4-Ig can disturb the migratory capacity of monocytes (Bonelli et al. 2016) and reduce the release of inflammatory cytokines, such as IFNγ and TNF-α, in rheumatoid arthritis patients (Pieper et al. 2013). CTLA4-Ig also promotes apoptosis of human monocytes (Tono et al. 2017). In addition to blocking CD80/86, CTLA4-Ig also decreases the expression of CD80, which disrupts co-stimulation of

inflammatory cells (Tono et al. 2017). However, the role and efficacy of CTLA4-Ig treatment in dystrophic muscle, especially in conjunction with other currently used medications, including prednisone, are unknown.

Based on our understanding of the beneficial effects by CTLA4-Ig in other inflammatory diseases and the success of prednisone in controlling inflammation in DMD, we tested if the effects achieved by treatment with CTLA4-Ig alone are influenced by co-administration with prednisone. We analyzed the combination of these two beneficial drugs, because it is possible that two drugs that reduce disease pathology can negatively interact together, resulting in a harmful outcome. For example, the combination of aspirin and clopidogrel increases mortality in mice with metastatic breast-cancer (Smeda et. al. 2018). Although the combination of these two treatments is a typical regiment in cardiovascular patients, these treatments harmfully affected cardiovascular function in mice with breast cancer (Smeda et. al. 2018). Therefore, we explored the combination of prednisone and CTLA4-Ig treatment to analyze if they were both effective in reducing *mdx* pathophysiology.

Materials and Methods

Animals

Experiments with mice were conducted according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the UCLA Institutional Animal Care and Use Committee. *Mdx* mice were purchased from the Jackson Laboratory and housed in a specific pathogen-free vivarium.

Mdx mice received daily, intraperitoneal injections with phosphate-buffered saline (PBS) or prednisone at 14 to18 and 21 to 25 days of age using 75 μg of prednisone per gram body weight. Injections of CTLA4-Ig (BioXCell) and isotype control antibody occurred on days 23, 25, and 27 using 25 μg per gram body weight. Mice were sacrificed at 4-weeks of age and the right hindlimb muscles were embedded in Tissue Tek O.C.T. and stored at -80^{o}C in vials filled with isopentane. Left hindlimb muscles were stored in cryovials in a storage tank containing liquid nitrogen.

Immunohistochemistry

Quadriceps muscles were sectioned at 10-μm thickness. Sectioned tissues were air dried for 30 minutes and sections were fixed with cold acetone (or 2% paraformaldehyde for FOXP3) for 10 minutes. Sections were air dried again for 10 minutes, washed in PBS for 5 minutes, quenched with 0.3% H_2O_2 in PBS for 10 minutes, and washed in PBS for 5 minutes. The slides were incubated with blocking buffer containing 3% bovine serum albumin, 0.2% gelatin and 0.05% Tween-20. Tissues were subsequently washed with PBS and incubated with primary antibody diluted in antibody buffer solution overnight at 4^{o}C (rat anti-F4/80 [eBioscience, 1:100], rat anti-

CD206 [BioRad 1:50], hamster anti-CD80 [Biolegend, 1:50], goat anti-collagen type I [Southern Biotech, 1:50], goat anti-collagen type III (Southern Biotech, 1:50), or goat anti-collagen type V [Southern Biotech, 1:50]) or for 3 hours at room temperature (rat anti-CD68 [Serotec 1:100], rabbit anti-FOXP3 [Abcam, 1:50], rat anti-CD8 [Southern Biotech, 1:100], or rabbit anti-CD163 [Santa Cruz Biotechnology, 1:100]).

After incubation with the primary antibody, sections were incubated with host-specific secondary antibody for 30 minutes, washed three times in PBS, and incubated with avidin-D horseradish peroxidase (HRP) for 30 minutes. Labeling was then visualized with AEC substrate kit, mounted with VectaMount (Vector Laboratories), and stored at -20^{o}C.

Injured fibers were determined via labeling with anti-albumin. The protocol described above was used for anti-albumin immunolabeling of sections except muscles were fixed in cold methanol and no secondary antibody was used. Sections were incubated with rabbit anti-albumin primary antibody conjugated to avidin-D HRP overnight at 4^{o}C (Nordic Immunology, 1:20) and developed using an AEC kit the following day. The number of stained muscle fibers were counted and divided by the total number of fibers to calculate the percentage of injured fibers.

Macrophages and T-cells were quantified using an Olympus BX50 microscope with a 40X objective lens and a 10 x 10 micrometer grid. The number of cells per volume was determined by counting the number of labeled cells divided by the total number of grid intercepts with overlying tissue. This number was then multiplied by the section thickness to calculate cells per unit volume. Macrophage infiltration was quantified as the number of labeled cells inside the

muscle fibers and macrophages in connective tissue were quantified as the number of labeled cells inside the perimysium and endomysium surrounding muscle fibers.

Collagen accumulation was quantified using an Olympus BX50 microscope with a 20x objective lens. The number of intercepts overlying tissue stained with anti-collagen I, III, or V were counted and divided by the total number of intercepts to calculate the percent area of the section that was occupied by collagen.

Cross-sectional area (CSA):

Quadriceps were sectioned at 10-μm thickness and stained with filtered hematoxylin (Gill's formula, Vector Laboratories). Fiber CSA for each muscle was quantified as the average CSA of 500 myofibers in each quadriceps. CSA was calculated with Bioquant Image Analysis software at a magnification of 10x and fiber area was calculated on ImageJ.

RNA isolation

For each sample, the left quadriceps muscle was excised and homogenized in TRIzol (Life Technologies) using a Sorvall omni-mixer. Samples were kept on ice and homogenized three times for 30-second periods separated by 20-second intervals. Homogenized tissues were centrifuged at 12,000 g for 10 minutes at 4^{o}C to remove insoluble material and incubated at room temperature to allow for dissociation of the nucleoprotein complex. Chloroform at 20% of the original volume of TRIzol was then added to each sample. Samples were agitated for 15 seconds, incubated for 3 minutes, and then centrifuged for 12,000 g for 15 minutes at 4^{o}C to separate the three layers: organic phenol-chloroform, protein, and RNA. The layer of RNA was carefully

extracted, transferred into new tubes, and mixed with isopropanol at 50% of the original TRIzol volume. Samples were incubated at room temperature for 10 minutes at then centrifuged at 12,000 g for 10 minutes at 4°C. The remaining RNA pellet was washed with 75% ethanol and centrifuged at 7,500 g for 5 minutes at 4°C. The supernatant was then removed and the pellet was air dried on ice for 5 minutes. Pellets were resuspended in water and the RNA was purified using the RNeasy Mini Kit (Qiagen). RNA concentration was determined via a Beckman DU730 spectrophotometer (Beckman Coulter). Samples were analyzed at wavelengths of 260 and 280 nm and all RNA samples had a 260/280 ratio above 2.0. RNA quality was visualized on a 1.2% agarose gel.

Quantitative real-time polymerase chain reaction (qPCR)

A master-mix containing Oligo(dT) 12-18 primer (Invitrogen) and 10 mM dNTPs was added to 2 µg of RNA. The samples were then heated to 65°C for 5 minutes. A second master-mix containing First-Strand Buffer (Invitrogen), 0.1M dithiothreitol (Invitrogen), SuperScript™ II RT (Invitrogen), and autoclaved water was added to each sample. To complete the reverse transcription, the samples were incubated at 42°C for 50 minutes, heated to 70°C for 50 minutes, and diluted 1:1 in autoclaved water to be stored at -20°C.

QPCR experiments were performed using a 96-well plate with each well containing 2 µl of cDNA and 23 µl of a master-mix that included autoclaved water, SYBR® Green Supermix (Bio-Rad) and the appropriate forward and reverse primers for the genes of interest (Table 1) with the exception of IL-4 that contained 4 µl of cDNA and 21 µl of master-mix in each well. The

reference genes used were Srp14 and Rpl13a for all samples. Experiments were performed on a Quantstudio 5 Real-Time PCR System (Thermo Fisher).

Statistical Analysis

All statistical analyses were performed using one-way ANOVA with differences considered significant at $p < 0.05$. All graphs display the mean ± the standard error of the mean (SEM).

Gene	**5' to 3' Sequence**
Col1α1 **F**	TGTGTGCGATGACGTGCAAT
Col1α1 **R**	GGGTCCCTCGACTCCTACA
Col3α1 **F**	ATCCCATTTGGAGAATGTTGTGC
Col3α1 **R**	GGACATGATTCACAGATTCCAGG
Col5α3 **F**	CGGGGTACTCCTGGTCCTAC
Col5α3 **R**	GCATCCCTACTTCCCCCTTG
FOXP3 **F**	CTTTCACCTATGCCCACCCTTATC
FOXP3 **R**	TAGATTTCATTGAGTGTCCTCTGC
CD8α **F**	ATGGCTTCATCCCACAACAAG
CD8α **R**	CGTGTCCCTCATGGCAGAA
IL-10 **F**	CAAGGAGCATTTGAATTCCC
IL-10 **R**	GGCCTTGTAGACACCTTGGTC
IFNγ **F**	GACAATCAGGCCATCAGCAAC
IFNγ **R**	CGGATGAGCTCATTGAATGCTT
TNFα **F**	CTTCTGTCTACTGAACTTCGGG

TNFα **R**	CACTTGGTGGTTTGCTACGAC
IL-4 **F**	GGATGTGCCAAACGTCCTC
IL-4 **R**	GAGTTCTTCTTCAAGCATGGAG
Srp14 **(HKG) F**	AGAGCGAGCAGTTCCTGAC
Srp14 **(HKG) R**	CGGTGCTGATCTTCCTTTTC
Rpl13a **(HKG) F**	CCTGCTGCTCTCAAGGTTGTT
Rpl13a **(HKG) R**	CGATAGTGCATCTTGGCCTTT

Table 1. Primer Sequences (F – Forward primer; R – Reverse primer; HKG – Housekeeping genes)

Results

Co-treatment with prednisone and CTLA4-Ig reduces muscle inflammation

We first analyzed the effects of prednisone and CTLA4-Ig on inflammatory cell numbers and inflammation-related cytokines. We initially examined the presence of cells expressing F4/80, which is a glycoprotein located on the surface of murine macrophages and is a marker used to identify macrophages in tissues (McKnight et al. 1996). Our results show that treatment with prednisone and control Ig (Pred-Ig treatment) or co-treatment with prednisone and CTLA4-Ig (Pred/CTLA4-Ig treatment) similarly reduced F4/80+ cells (Fig. 1A-D). We also found that only the Pred/CTLA4-Ig treatment reduced the cell density of pro-inflammatory CD68+ macrophages (Fig. 1E-H). Pred/CTLA4-Ig treatment also reduced the number of CD68+ macrophages within muscle fibers (Fig. 2A, B) and within the connective tissue (Fig. 2C, D). Quadriceps muscles of mice treated with the Pred/CTLA4-Ig treatment also showed no difference in numbers of anti-inflammatory CD206+ macrophages compared to the control group (Fig. 3A-C). We also tested for the presence of CD80+ cells, a ligand located on the surface of antigen-presenting cells that participate in co-stimulation of T-cells (Gardner et al. 2014). and found that only the Pred/CTLA4-Ig treatment reduced the number of CD80+ cells compared to controls (Fig. 3D, E). This shows a CTLA4-Ig specific effect on reducing the prevalence of antigen-presenting cells.

We also examined the effects of prednisone and CTLA4-Ig treatment on lymphoid cells because cytotoxic CD8+ T-cells exacerbate dystrophic pathology through release of cytotoxic granules and apoptosis, (Spencer et al. 1997). Pred-Ig treatment was previously shown to diminish numbers of CD8+ cells (Wehling-Henricks et al. 2004). Similarly, we found that both Pred-Ig treatment and Pred/CTLA4-Ig treatment reduced CD8+ T-cell density compared to controls (Fig.

4A, B). Neither treatment regimen significantly affected the expression levels of CD8α (Fig. 4E), a component of the transmembrane glycoprotein CD8 that plays a role in aiding cytotoxic T-cell interactions (Hennecke et al. 1993). We also analyzed the effects of the treatments on FOXP3+ regulatory T-cells, which could potentially reduce dystrophic pathology by promoting T-cell homeostasis (Villalta et al. 2014). However, we found that neither treatment significantly affected FOXP3+ cell density compared to controls (Fig. 4C, D). Surprisingly, Pred/CTLA4-Ig treatment significantly reduced FOXP3 expression levels compared to control and Pred-Ig treatment groups, indicating a CTLA4-Ig specific reduction (Fig 4F).

We then examined the effects of Pred/CTLA4-Ig treatment on relative mRNA expression levels of pro-inflammatory and anti-inflammatory cytokines. IFNγ and TNF-α are pro-inflammatory cytokines that activate M1 macrophages (Pechold et al. 1997). Our findings show that Pred/CTLA4-Ig treatment decreased the IFNγ expression (Fig. 5B) levels compared to controls, but did not significantly affect TNF-α expression (Fig. 5C). IL-4 and IL-10 are anti-inflammatory cytokines that activate M2 macrophages (Villalta et al. 2011). Neither treatment altered expression levels of either anti-inflammatory cytokine IL-10 (Fig. 5A) or IL-4 (Fig. 5D), which parallels the lack of treatment effects on the numbers of CD206+ M2 macrophages.

Pred/CTLA4-Ig treatment decreases necrosis of muscle fibers in *mdx* quadriceps

We also assayed for effects of the treatments on muscle damage because our previous findings showed that reduction in myeloid or lymphoid cell populations in *mdx* muscles reduced muscle damage (Wehling et al., 2001; Villalta et al. 2009). M1 pro-inflammatory macrophages and cytotoxic T-cells contribute to muscle necrosis, both of which are decreased with Pred-Ig or

Pred/CTLA4-Ig treatment. Our findings show that Pred-Ig and Pred/CTLA4-Ig treatments significantly diminished the number of injured fibers compared to controls (Fig. 6A-D). The Pred/CTLA4-Ig treatment also further decreased albumin-positive fibers compared to Pred-Ig treatment (Fig. 6A). Therefore, the Pred/CTLA4-Ig treatment was the most effective in reducing the number of injured fibers.

Pred/CTLA4-Ig treatment reduces collagen accumulation and reduces pro-fibrotic macrophages

Previous work from our lab demonstrated that reduced inflammation led to reduced fibrosis in *mdx* dystrophic muscles (Wehling-Henricks et al. 2010), so we next analyzed the effects of the treatments on muscle fibrosis and pro-fibrotic macrophages. We found that Pred/CTLA4-Ig treatment did not significantly affect collagen I (Fig. 7A, D) or III accumulation (Fig. 7B, E), but both Pred-Ig and Pred/CTLA4-Ig treatments significantly reduced collagen V accumulation in 4-week *mdx* quadriceps (Fig. 7C, F). Levels of collagen V also differed between Pred-Ig and the Pred/CTLA4-Ig treatment groups with Pred/CTLA4-Ig having a more significant effect (Fig. 7C). We then examined the effect of treatment on the fibrosis associated M2 macrophage marker, CD163 (Kazanov et al. 2014). Our findings show that both the Pred-Ig and the Pred/CTLA4-Ig treatment significantly reduced CD163+ macrophage density compared to controls (Fig. 8A-D). Because pro-fibrotic M2 macrophages play a role in exacerbating dystrophic pathology (Wehling-Henricks et al. 2010), prednisone and CTLA4-Ig treatment reductions in CD163+ cells could potentially reduce fibrosis in *mdx* tissue.

We also analyzed the effects of treatment on relative mRNA expression levels of collagens I, III, and V (Fig. 9A-C). Interestingly, Pred/CTLA4-Ig treatment reduced the expression of collagen I and III compared to Pred-Ig treatment only (Fig. 9A, B). Pred/CTLA4-Ig treatment also decreased collagen V expression in 4-week *mdx* quadriceps compared to the control group (Fig. 9C).

Pred/CTLA4-Ig treatment does not affect myogenesis

Although we saw that Pred/CTLA4-Ig treatment reduced inflammation, necrosis, and collagen accumulation, we found that the Pred/CTLA4-Ig treatment had no effect on myogenesis in 4-week *mdx* quadriceps (Fig. 10A-G). Treatment effects were determined by CSA analysis, which is an indicator of muscle regeneration. Variance was also calculated to evaluate the distribution of fibers, but Pred-Ig treatment and Pred/CTLA4-Ig treatment displayed no effect on the CSA or their variances.

Discussion

In this study, we analyzed whether the beneficial effects that we had achieved previously by treating *mdx* mice with CTLA4-Ig were affected by prednisone co-administration. Although the specific mechanism by which prednisone and CTLA4-Ig reduce dystrophic pathology is unknown, our results indicate that administration of prednisone and CTLA4-Ig together can modulate the immune system and successfully reduce pathology in *mdx* muscles.

Our findings showed that Pred/CTLA4-Ig treatment reduced specific macrophage phenotypes in *mdx* muscles. These macrophage populations include M1 macrophages that enhance tissue damage and other M2 macrophages that promote fibrosis (Villalta et al. 2009). We showed that Pred/CTLA4-Ig treatment reduced the numbers of M1 CD68+ macrophages. A reduction in this cell population could potentially alleviate dystrophic pathology. We demonstrated previously that CTLA4-Ig reduces CD68+ macrophages in 4-week *mdx* quadriceps (Kok 2017), and the reduction of this M1 macrophage population is not affected by the addition of prednisone. Pred/CTLA4-Ig and Pred-Ig treatment also reduced the number of F4/80+ macrophages. However, Pred-Ig treatment did not reduce the CD68+ cell density, meaning that the reduction in CD68+ macrophages may be CTLA4-Ig specific. This also suggests that F4/80, the surface glycoprotein on differentiated murine macrophages, may be a pan macrophage marker and that there is a population of F4/80+ macrophages that is not CD68+. Similar to our results, CTLA4-Ig treatment reduced M1 macrophages in mice with inflammatory dermal fibrosis (Ponsoye et al. 2016). We also showed that Pred/CTLA4-Ig treatment reduced the number of CD68+ cells within the connective tissue and the fibers, thereby affecting the distribution of CD68+ cells as

well. Again, the effect of CTLA4-Ig on distribution of CD68+ macrophages was unaffected by the combination with prednisone.

Pred-Ig and Pred/CTLA4-Ig treatments did not affect CD206+ M2 macrophage numbers, but did reduce the number of CD163+ M2 macrophages. This could be due to the different effects by distinct M2 macrophage populations, as CD163+ macrophages promote fibrogenesis and CD206+ macrophages enhance regeneration (Villalta et al. 2009, Villalta et al. 2011). Thus, a reduction in the CD163+ macrophage population may have influenced the reduction in collagen accumulation and the maintenance of CD206+ M2 macrophages may have influenced the stable rate of myogenesis. These results also suggest that CTLA4-Ig treatment may polarize macrophages to a M2 phenotype rather than to a M1 pro-inflammatory phenotype that normally comprises the macrophage population at 4-weeks. These differential effects on different M2 macrophage subtypes by CTLA4-Ig were also unaffected by co-administration with prednisone, as we showed previously that CTLA4-Ig treatment alone had a similar outcome (Kok 2017, Kannan 2021).

Pred/CTLA4-Ig treatment also reduced the percentage of injured fibers in the *mdx* quadriceps. Thus, the reduced necrosis may be due to the reduced invasion of M1 pro-inflammatory macrophages in muscle fibers. Additionally, the reduced necrosis could be from the reduced CD8+ cell density and the strong trend in reduced expression of CD8α from the Pred/CTLA4-Ig treatment. CTLA4-Ig treatment showed similar effects on CD8+ cell activity in mice with graft versus host disease (Young et al. 2017). Our lab also showed that CTLA4-Ig treatment alone reduced CD8α expression in 4-week *mdx* quadriceps (Kok 2017). Thus, the reduction of CD8α

by CTLA4-Ig in 4-week *mdx* quadriceps was not disrupted by combination with prednisone. CTLA4-Ig may directly block the activation and proliferation of cytotoxic T-cells because CTLA4-Ig blocks co-stimulation of CD28 by CD80 between antigen presenting cells and naïve T-cells (Herrero-Beaumont et al. 2012). Therefore, Pred/CTLA4-Ig treatment may further reduce activation of CD8+ cells by reducing the number of CD80+ cells within 4-week *mdx* quadriceps. Our lab showed that CTLA4-Ig treatment alone reduced numbers of CD80+ cells in 4-week *mdx* quadriceps (Kok 2017). Although Pred-Ig treatment did not decrease numbers of CD80+ cells, CTLA4-Ig still reduced the number of CD80+ cells even when co-administered with prednisone. CTLA4-Ig also downregulated the expression of CD80 on primary cultures of macrophages from patients with rheumatoid arthritis (Cutolo et al. 2013), thereby displaying similar effects in other inflammatory diseases.

The reduction in CD163+ macrophages is consistent with the reductions in collagen V accumulation that resulted from Pred-Ig and Pred/CTLA4-Ig treatments. qPCR analysis also showed that Pred/CTLA4-Ig treatment reduced collagen V mRNA expression. Neither Pred-Ig or Pred/CTLA4-Ig treatment reduced collagen I or III accumulation, but Pred/CTLA4-Ig reduced collagen I and III expression compared to Pred-Ig treatment. Thus, Pred-Ig and Pred/CTLA4-Ig exert differential effects on collagen expression and even when CTLA4-Ig is co-administered with prednisone, the beneficial effects on reducing fibrosis still remain. The interaction between collagens I and V is necessary for the development of fibrils in the connective tissue, so the reduction in collagen V expression and accumulation may disrupt the structure of collagen I (Birk et al. 1990). Pred/CTLA4-Ig treatment is the most effective in ensuring the reduction of collagen accumulation and expression. CTLA4-Ig treatment showed similar effects in mice with

induced dermal fibrosis, as CTLA4-Ig caused regression of established dermal fibrosis and reduced the occurrence of novel fibrosis (Ponsoye et al. 2016).

We analyzed the effects of Pred/CTLA4-Ig treatment on the expression of selected cytokines, to better understand the differential results on various subpopulations of macrophages. Both Pred-Ig and Pred/CTLA4-Ig treatments reduced IFNγ expression. Our lab previously showed similar effects of CTLA4-Ig treatment alone on IFNγ expression in 4-week *mdx* quadriceps (Kok 2017), and the co-administration with prednisone amplified the effect. In the present study, CTLA4-Ig treatment produced a strong trend in reducing IFNγ expression, but Pred/CTLA4-Ig treatment resulted in a significant decrease in IFNγ expression. This result also reflects the reduction in number of CD68+ macrophages, as IFNγ activates M1 pro-inflammatory macrophages (Villalta et al. 2011). The reduction in IFNγ could also be due to reduction in CD8+ cells, since CD8+ T-cells can release IFNγ (Bhat et al. 2017). CTLA4-Ig treatment also reduced IFNγ expression in patients with rheumatoid arthritis (Pieper et al. 2013). CTLA4-Ig treatment also did not significantly alter expression of TNF-α or anti-inflammatory cytokines IL-4 or IL-10, similar to the Pred/CTLA4-Ig treatment. However, CTLA4-Ig treatment reduced expression of TNF-α in patients with rheumatoid arthritis, which is potentially beneficial because TNF-α can be responsible for events leading to necrosis or apoptosis (Cutolo et al. 2013). The lack of effect of Pred-Ig or Pred/CTLA4-Ig treatments on IL-4 and IL-10 anti-inflammatory cytokines may underlie the lack of effect of the treatments on CD206+ cell density because those cytokines can drive macrophages to a CD206+ phenotype (Villalta et al. 2011, Casella et al. 2016). This negative outcome differs from the influence of CTLA4-Ig treatment on expression of IL-4 and

IL-10 in mice with hypersensitivity pneumonitis, in which CTLA4-Ig reduced expression of these cytokines (Israël-Assayag 1999).

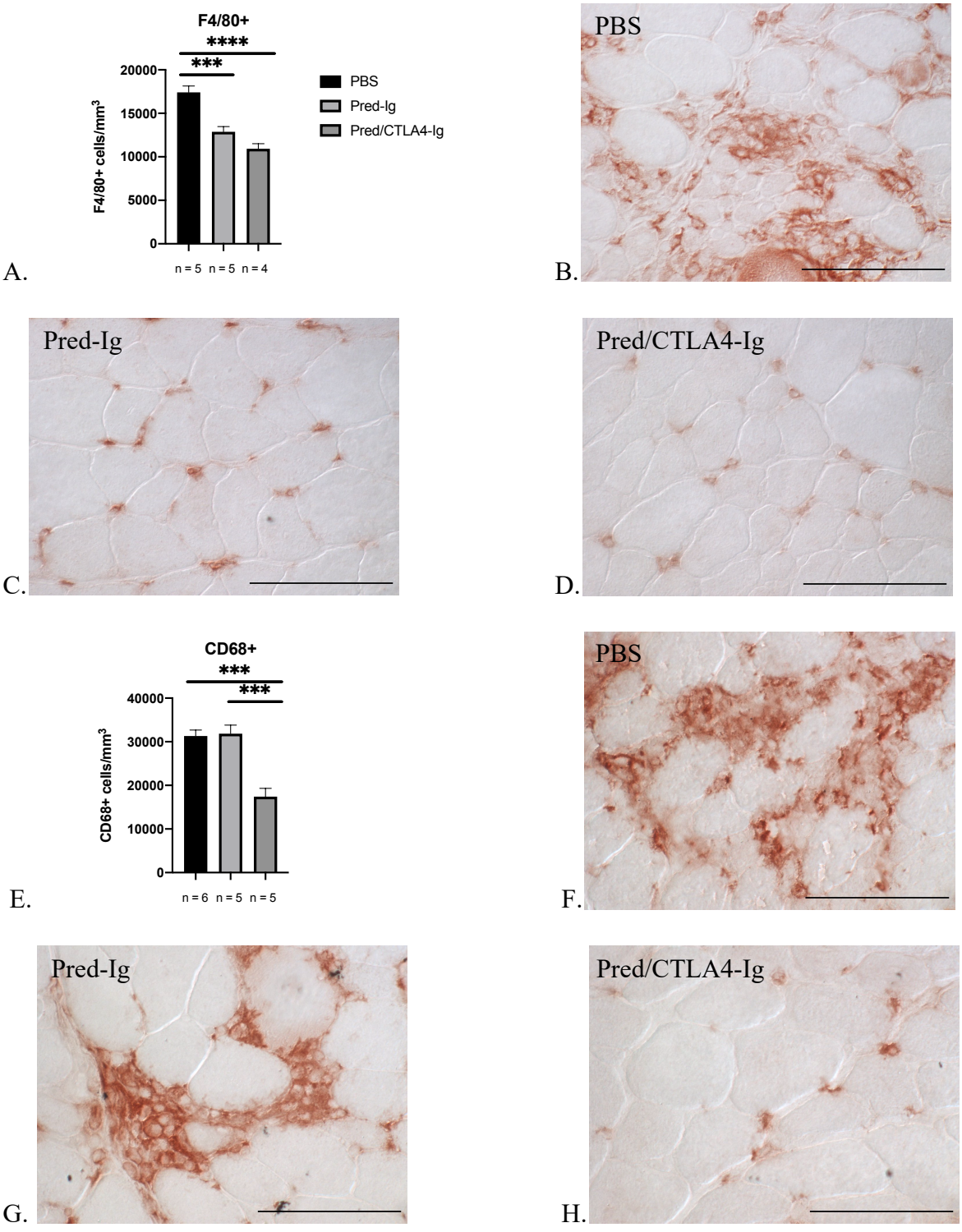
Regulatory T-cells are a major source of IL-10 and we found that Pred/CTLA4-Ig treatment did not affect FOXP3+ cell density (Villalta et al. 2014). However, we saw that Pred/CTLA4-Ig treatment significantly reduced the expression of FOXP3. Therefore, the mRNA expression did not reflect the protein levels. Since the FOXP3 transcription factor maintains CD4+ T-cells in a regulatory T-cell state, the amount of FOXP3 mRNA or protein may not determine the efficiency of regulatory T-cells on immune homeostasis (Zhang et al. 2007). Our lab showed previously that CTLA4-Ig treatment alone reduced expression of FOXP3 mRNA in 4-week *mdx* quadriceps (Kok 2017) and the addition of prednisone in the current investigation did not change that outcome. CTLA4-Ig treatment alone also produced a strong trend for decreasing FOXP3+ cell numbers in 4-week quadriceps (Kok 2017). Therefore, another explanation may be that the recruitment of regulatory T-cells is unnecessary because CTLA4-Ig exerts similar effects in maintaining T-cell homeostasis (Deppong et al. 2013). CTLA4-Ig treatment similarly affects FOXP3 expression in patients with rheumatoid arthritis (Pieper et al. 2013). Additionally, the proliferation of regulatory T-cells may be dependent on the presence of antigen-presenting cells and we showed that Pred/CTLA4-Ig reduced the number of CD80+ cells (Razmara et al. 2008).

Our lab also showed previously that CTLA4-Ig treatment on 4-week *mdx* quadriceps did not affect myogenesis (Kok 2017). Neither Pred/CTLA4-Ig or CTLA4-Ig treatment alone displayed any effect on mean CSA or variance (Kok 2017). Thus, even with the addition of prednisone,

CTLA4-Ig treatment did not affect fiber size. Since Pred/CTLA4-Ig did not affect myogenesis, the beneficial effects on necrosis and inflammation are not due to enhanced myogenesis in the muscles.

In conclusion, our results show that CTLA4-Ig may be a novel immunotherapy to reduce pathology in DMD. Prednisone and CTLA4-Ig can also be utilized together to effectively diminish inflammation, necrosis, and fibrosis in *mdx* tissue and the beneficial effects of CTLA4-Ig remain enhanced or unaffected by combination with prednisone. Future experiments should explore the combination of intermittent prednisone dosing and CTLA4-Ig, as this may diminish some of the harmful side effects that accompany long-term use of prednisone (Quattrocelli et al. 2017).

FIGURE 1



<u>Figure 1.</u> Pred-Ig and Pred/CTLA4-Ig treatments diminished muscle inflammation in 4-week *mdx* quadriceps. **(A)** F4/80+ macrophage density was reduced with Pred-Ig and Pred/CTLA4-Ig treatments (*** indicates $p < 0.001$, **** indicates $p < 0.0001$). **(B-D)** Representative images of

4-week *mdx* quadriceps show decreased presence of F4/80+ macrophages in Pred-Ig (C) and Pred/CTLA4-Ig (D) treatment groups. **(E)** CD68+ macrophage density is reduced with Pred/CTLA4-Ig treatment compared to both control and Pred-Ig treated groups. **(F-H)** Representative images of CD68+ macrophages in quadriceps muscle at 4-weeks of age in PBS (F), Pred-Ig (G) and Pred/CTLA4-Ig (H) treatment groups. Data are presented as mean ± SEM. P values are based on one-way ANOVA test. Scale bars = 100 μm.

FIGURE 2:

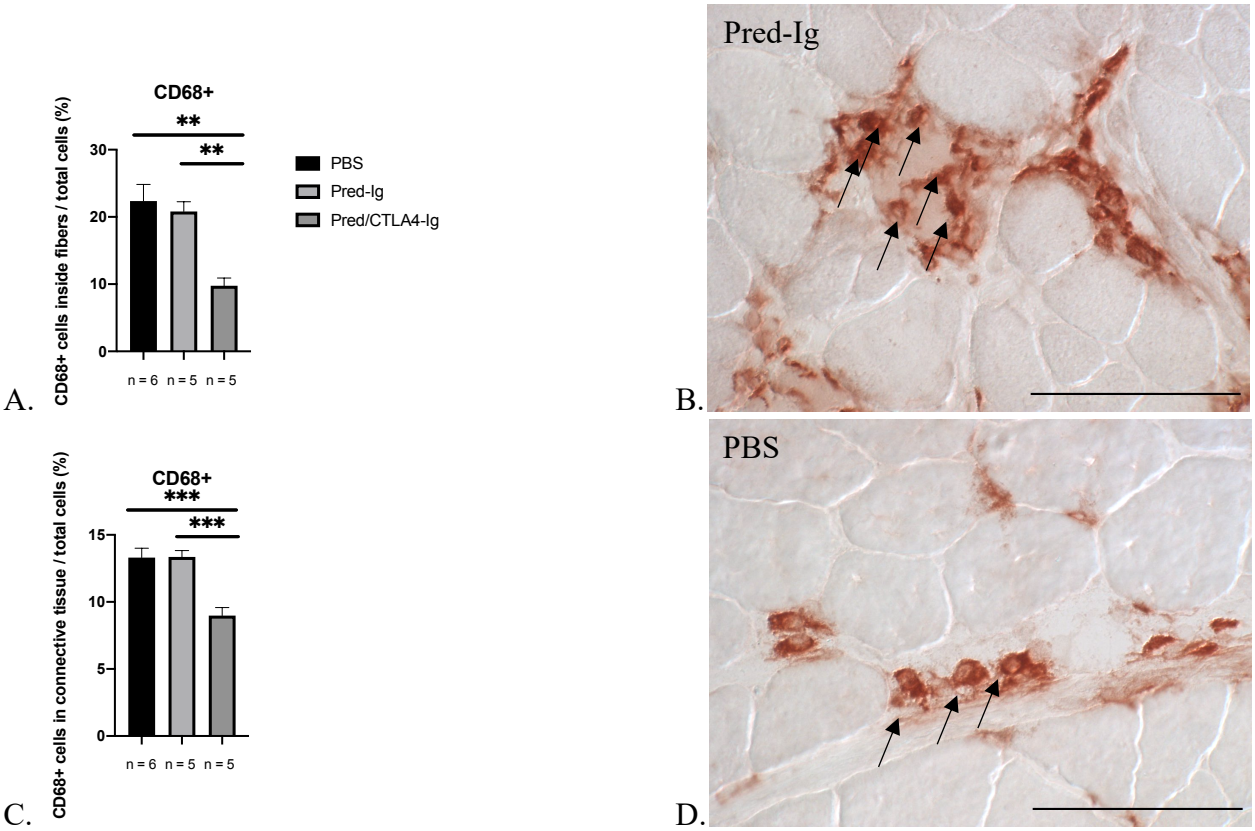
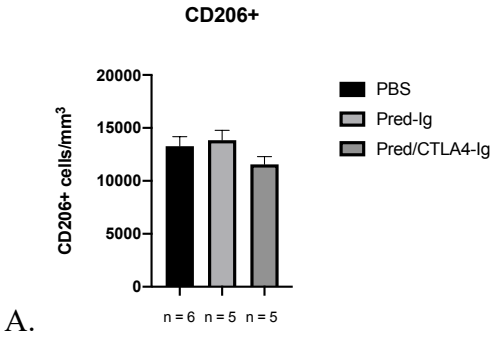
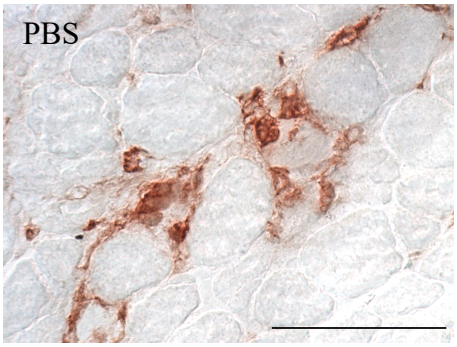


Figure 2. Pred/CTLA4-Ig affected the distribution of CD68+ macrophages in 4-week *mdx* quadriceps muscle. (**A, C)** Pred/CTLA4-Ig reduced accumulation of CD68+ macrophages in muscle fibers (A) and in connective tissue (C) (** indicates $p < 0.01$, *** indicates $p < 0.001$). **(B, D)** Representative images of CD68+ macrophages in a necrotic fiber and the connective tissue of a quadriceps muscle at 4-weeks of age in Pred-Ig (B) and PBS (D) treatment groups. Arrows indicate positively-labelled cells. Data are presented as mean ± SEM. P values are based on one-way ANOVA test. Scale = 100 μm.

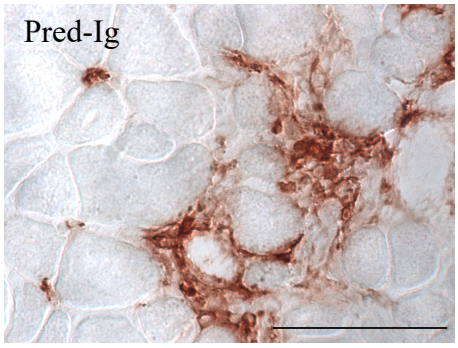
FIGURE 3:



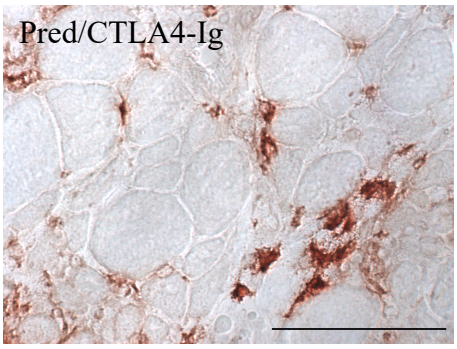
A.



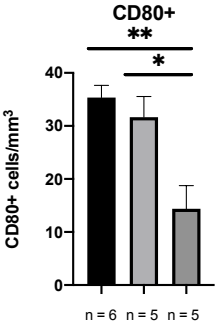
B.



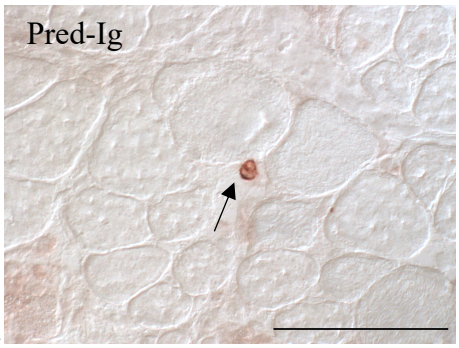
C.



D.



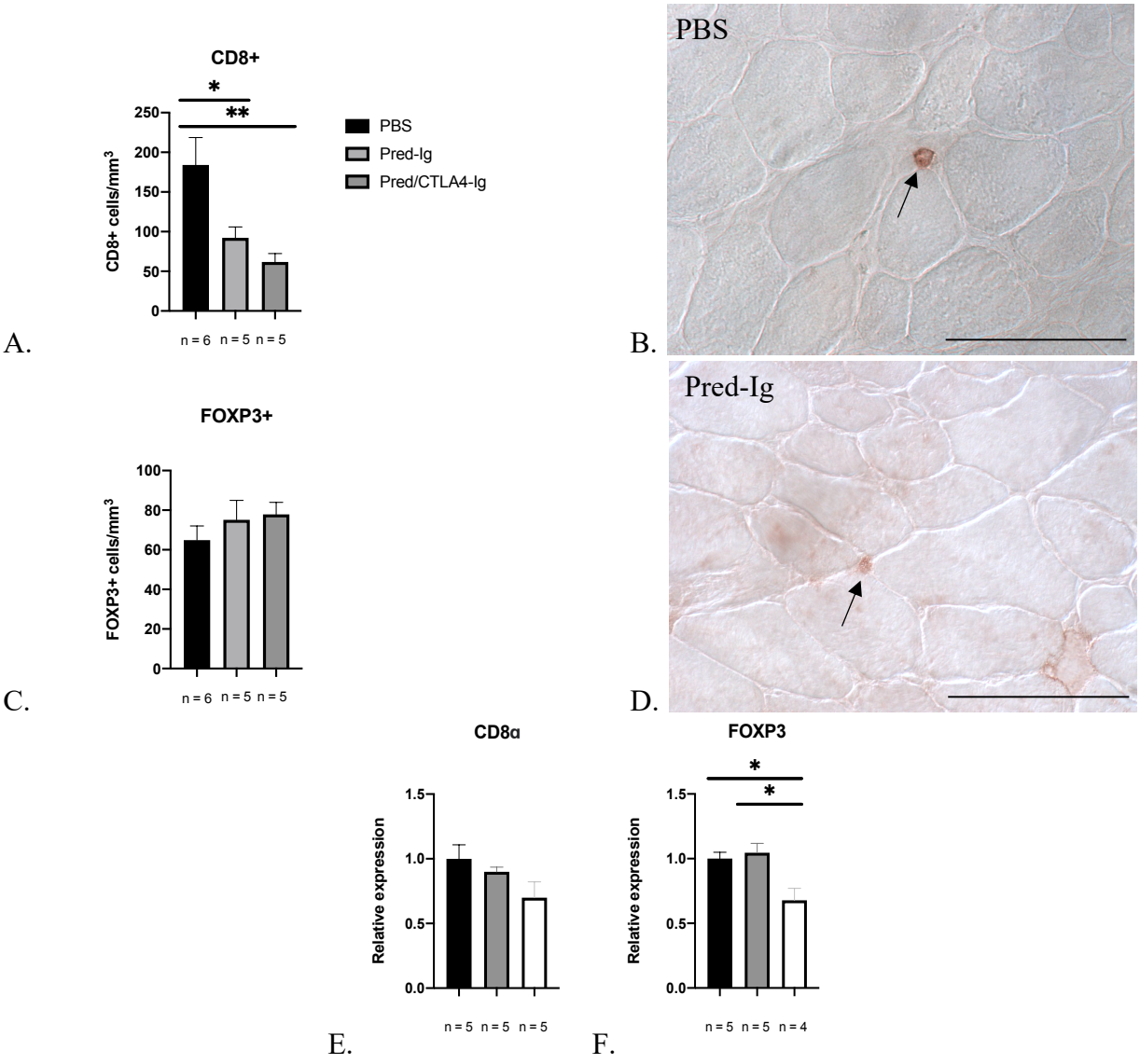
E.



F.

<u>Figure 3:</u> Pred/CTLA4-Ig treatment did not affect numbers of anti-inflammatory macrophages and selectively reduced CD80+ cells. **(A)** CD206+ macrophage density was unaffected by Pred-Ig or Pred/CTLA4-Ig. **(B-D)** Representative images show similar presence of CD206+ cells between all treatment groups. **(E)** Pred/CTLA4-Ig treatment reduces CD80+ cell density in the quadriceps muscles of 4-week *mdx* mice treated compared to the either control or Pred-Ig treated groups. **(F)** Representative image shows CD80+ cell in PBS-treated 4-week *mdx* quadriceps. Arrow indicates positively-labelled cell. Data are presented as mean ± SEM. P values are based on one-way ANOVA test. Scale = 100 μm.

FIGURE 4



<u>Figure 4.</u> Pred/CTLA4-Ig decreased cytotoxic T-cell density and did not affect regulatory T-cell density, neither of which were reflected by relative mRNA expression levels. (**A)** Reduced CD8+ T-cell density in Pred-Ig and Pred/CTLA4-Ig treated quadriceps muscle compared to control (* indicates $p < 0.05$, ** indicates $p < 0.01$). (**B)** Representative image of 4-week *mdx* quadriceps treated with PBS shows CD8+ cell. **(C)** No change in FOXP3+ cell density with either treatment group. **(D)** Representative image of 4-week *mdx* quadriceps treated with Pred-Ig shows FOXP3+ cell **(E-F)** Additionally, qPCR analysis of 4-week *mdx* quadriceps shows downward trend in **(E)** CD8α for co-treated mice and significant decrease in **(F)** FOXP3 expression in co-treated mice compared to control and Pred-Ig treated. Data are normalized to PBS control, set at "1" for each gene assayed. Arrows indicate positively-labelled cells. Data are presented as mean ± SEM. P values are based on one-way ANOVA. Scale bar = 100 μm.

FIGURE 5

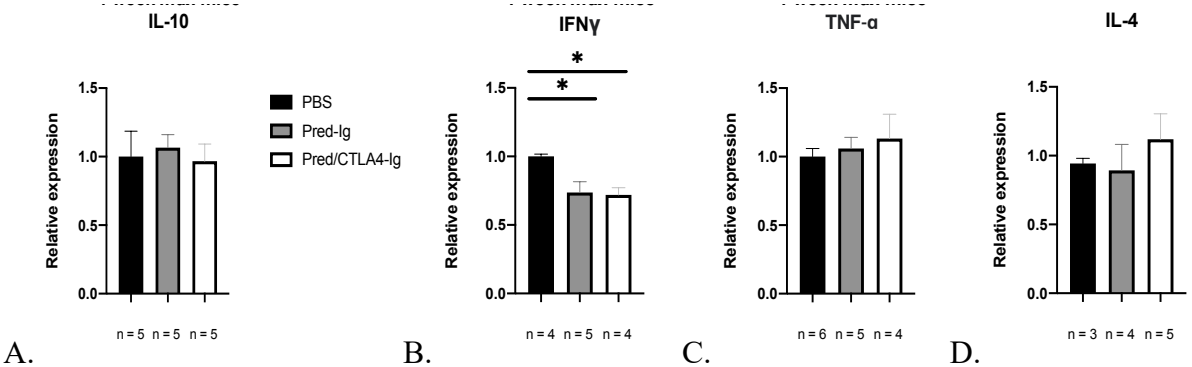


Figure 5. Pred-Ig and Pred/CTLA4-Ig treatments reduced expression of pro-inflammatory cytokine IFNγ and did not significantly affect expression of anti-inflammatory cytokines IL-4 or IL-10 or affect expression of TNF-α. qPCR analysis of 4-week *mdx* quadriceps shows no significant difference between any treatment groups for anti-inflammatory cytokine **(A)** IL-10, significant decrease between both treatment groups and control in (**B)** IFNγ expression, and no significant difference between either treatment group and control for expression of **(C)** TNF-α or **(D)** IL-4. Data are normalized to PBS control, set at "1" for each gene assayed. Data are presented as mean ± SEM. P values are based one-way ANOVA.

FIGURE 6

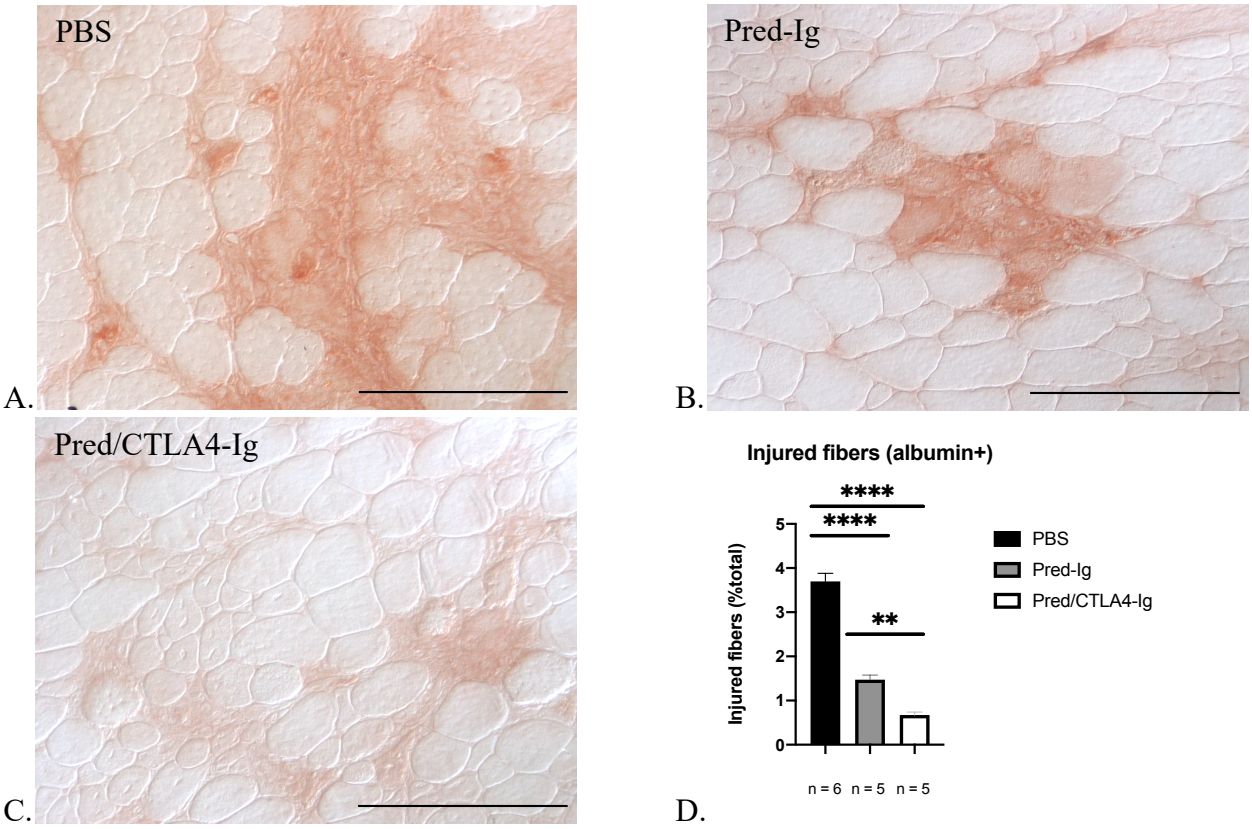


Figure 6. Pred/CTLA4-Ig reduced numbers of injured fibers in 4-week *mdx* quadriceps. **(A-C)** Representative images and **(D)** percentages of albumin-labelled fibers in 4-week-old *mdx* quadriceps treated with PBS, Pred-Ig, and Pred/CTLA4-Ig show large necrotic areas in the **(A)** PBS-treated samples and smaller necrotic areas with fewer necrotic fibers in the **(B)** Pred-Ig treated samples and **(C)** even fewer in the Pred/CTLA4-Ig treated samples (** indicates $p < 0.01$, **** indicates $p < 0.0001$). Data are presented as mean ± SEM. P values are based on one-way ANOVA. Scale bar = 100 μm.

FIGURE 7

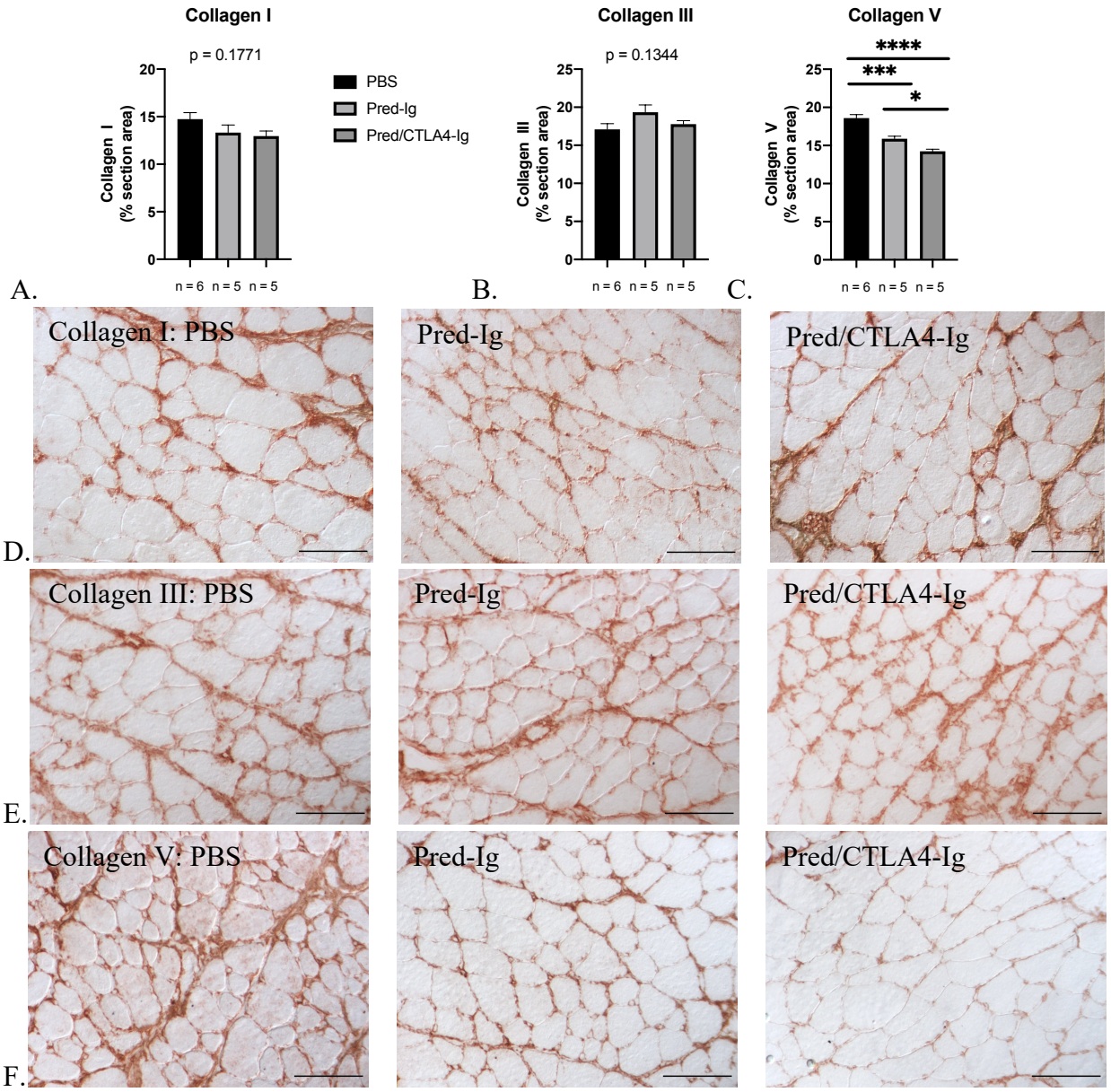


Figure 7. Pred-Ig and Pred/CTLA4-Ig selectively reduced collagen V accumulation in 4-week *mdx* quadriceps. **(A, B)** No significant effect on percent area of collagen I (A) or collagen III (B) for either treatment group. **(D, E)** Representative images for 4-week *mdx* quadriceps show similar accumulation of collagen I (D) and collagen III (E) for all treatments. **(C)** Both Pred-Ig and Pred/CTLA4-Ig reduce percent collagen V area compared to control and Pred/CTLA4-Ig further reduces collagen V area compared to Pred-Ig treatment alone (* indicates $p < 0.05$, *** indicates $p < 0.001$, **** indicates $p < 0.0001$). **(F)** Representative images show decreased collagen V accumulation with Pred-Ig treatment and further reduction in collagen V accumulation with Pred/CTLA4-Ig. Data are presented as mean ± SEM. P values are based on unpaired one-way ANOVA. Scale bar = 100 μm.

FIGURE 8

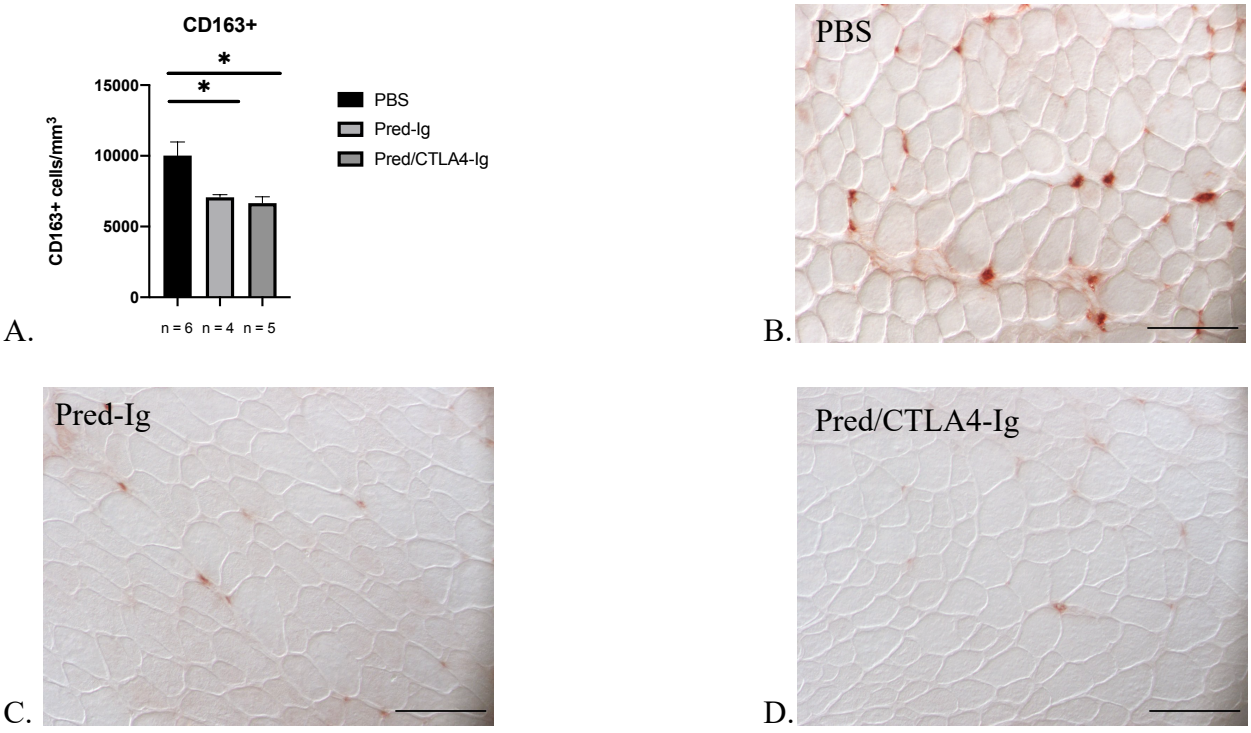
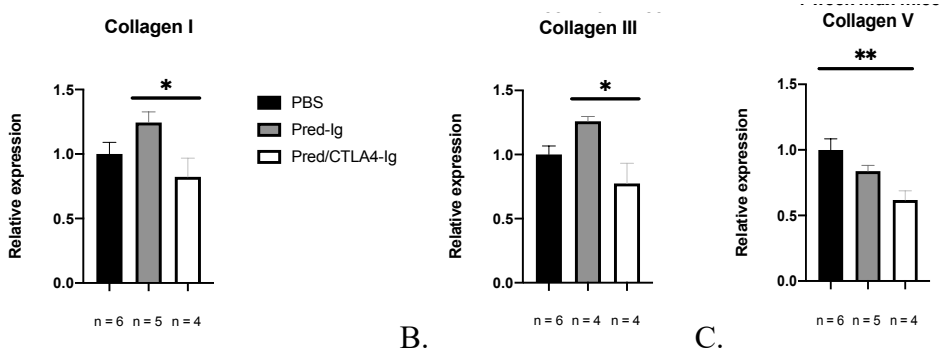


Figure 8. Pred-Ig and Pred/CTLA-4-Ig treatments reduced pro-fibrotic macrophages. **(A)** Pro-fibrotic CD163+ cell density is reduced with Pred-Ig and Pred/CTLA4-Ig. **(B-D)** Representative images show reduced CD163+ cell density in Pred-Ig (C) and Pred/CTLA4-Ig (D) groups compared to the control-treated group (B). Data are presented as mean ± SEM. P values are based on unpaired one-way ANOVA. Scale bar = 100 μm.

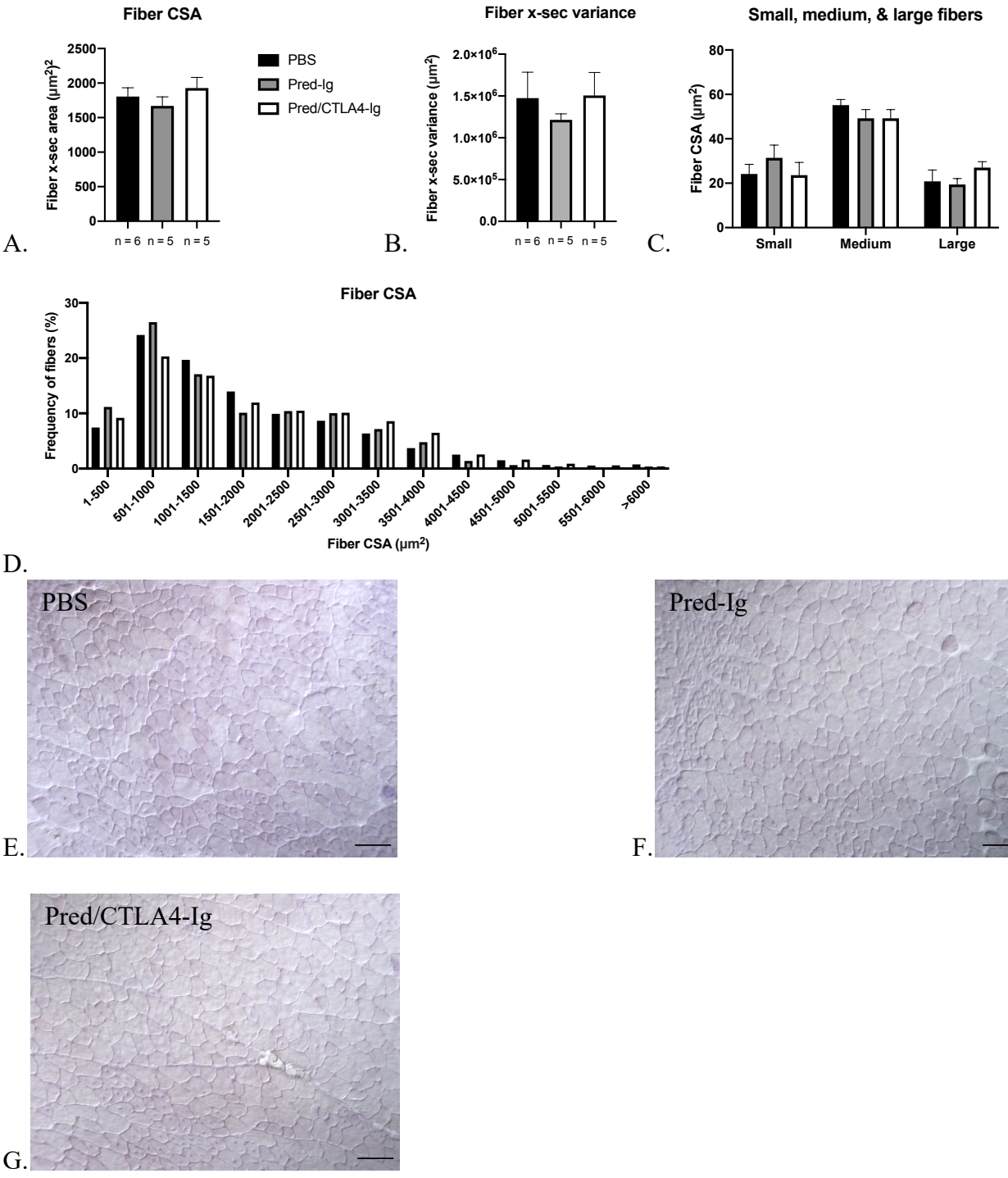
FIGURE 9



A. B. C.

Figure 9. Pred/CTLA4-Ig decreased relative mRNA expression of collagen I, III, and V. **(A-C)** qPCR analysis of 4-week *mdx* quadriceps shows Pred/CTLA4-Ig reduces expression of (**A)** collagen I and (**B)** collagen III compared to the Pred-Ig treated group. Additionally, qPCR analysis shows that the co-treatment reduces (**C)** collagen V expression compared to control mice (* indicates $p < 0.05$, ** indicates $p < 0.01$). Data are normalized to PBS control, set at "1" for each gene assayed. Data are presented as mean ± SEM. P values are based on unpaired one-way ANOVA.

FIGURE 10



<u>Figure 10.</u> Pred/CTLA4-Ig treatment did not affect myogenesis. **(A-D)** Muscle fiber CSAs were calculated for 500 myofibers/quadriceps. Co-treatment did not have a significant effect on (**A)** mean CSA (μm^2), **(B)** variance (μm^4), **(C)** distribution of small, medium, and large fibers (μm^2), or **(D)** frequency of fibers (%) for 4-week *mdx* quadriceps. **(E-G)** Representative images of 4-

week *mdx* quadriceps treated with **(E)** PBS, **(F)** Pred-Ig, or **(G)** Pred/CTLA4-Ig show similar distribution of fibers between treatment groups. Data are presented as mean ± SEM. P values are based on one-way ANOVA. Scale bar = 100 μm.

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