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Regulatory T cells suppress muscle inflammation and injury in muscular dystrophy

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Abstract

We examined the hypothesis that regulatory T cells (Tregs) modulate muscle injury and inflammation in the mdx mouse model of Duchenne muscular dystrophy (DMD). Although Tregs were largely absent in the muscle of wildtype mice and normal human muscle, they were present in necrotic lesions, displayed an activated phenotype and showed increased expression of interleukin (IL)-10 in dystrophic muscle from mdx mice. Depletion of Tregs exacerbated muscle injury and the severity of muscle inflammation, which was characterized by an enhanced interferon-gamma (IFNγ) response and activation of M1 macrophages. To test the therapeutic value of targeting Tregs in muscular dystrophy, we treated mdx mice with IL-2/anti-IL-2 complexes (IL-2c), and found that Tregs and IL-10 concentrations were increased in muscle, resulting in reduced expression of cyclooygenase-2 and decreased myofiber injury. These findings suggest that Tregs modulate the progression of muscular dystrophy by suppressing type 1 inflammation in muscle associated with muscle fiber injury, and highlight the potential of Treg-modulating agents as therapeutics for DMD.

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Author contributions.

SAV technically contributed to the experiments shown in Fig. 1–6, Fig. S1–S8, and Table S2. WR technically contributed to the experiment shown Fig. 3, 4, 5, 6, and Fig. S4, S5–S8. LM technically contributed to experiments shown in Fig. 1, 6, Fig. S8, and Table S2. AK performed genotyping of the mice used in this study, and performed flow cytometry and histological experiments not shown here. MM contributed to Fig. S8 and Table S1. TS provided mice and consultation regarding the DT-mediated depletion of Tregs in mdx^{DERG} mice. SAV, JGT, MJS and JAB designed all experiments. Data interpretation and assembly of the manuscript was performed by SAV and JAB. The manuscript was reviewed by each author before submission.

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Introduction

Duchenne muscular dystrophy (DMD) is a lethal muscle degenerative disorder that is caused by loss-of-function mutations in the dystrophin gene ([1, 2]). Hypomorphic dystrophin mutations result in a milder clinical phenotype known as Becker muscular dystrophy (BMD) ([3]). DMD is characterized by a progressive deterioration of muscle function and strength, loss of ambulation by the second decade of life and death in the early to mid-20s. Despite a kinetic difference in the progressive deterioration of muscle mass/function and the development of fatty fibrotic deposits between mdx mice and DMD/BMD patients, mdx mice similarly lack dystrophin and develop a muscular dystrophy that shares many pathological features with the human disease ([4]). Studies using the mdx mouse model have revealed that the physical and cellular basis of muscle degeneration can be attributed to loss of sarcolemmal integrity, increased susceptibility to oxidative stress, and severe myofiber degeneration and inflammation ([5–7]).

Although the lack of dystrophin protein is the primary defect responsible for the development of muscular dystrophy, secondary disease processes such as muscle inflammation contribute greatly to the pathogenesis of DMD ([8]). The leukocyte infiltrate of dystrophic muscle is heterogeneous comprising neutrophils, eosinophils, macrophages, and $CD8^+$ and $CD4^+$ T cells ([9–12]). Depletion of specific immune cell populations using anti-F4/80 or anti-Gr-1 (myeloid cell-specific), anti-CD4 or anti-CD8 (T cell-specific) antibodies has shown that reducing the numbers of myeloid cells or T cells in dystrophic muscle decreased the proportion of necrotic myofibers in mdx mice ([10–12]). Similarly, broad glucocorticosteroid-mediated immunosuppression in DMD patients and mdx mice delays the severity and progression of muscular dystrophy, likely involving suppression of muscle inflammation and direct effects on muscle function ([13–15]).

In addition to promoting muscle injury during muscular dystrophy, the immune system also facilitates muscle regeneration and repair ([16]). This dichotomous role of the immune system in muscle disease may be partly explained by the development of type 1 and type 2 inflammatory responses that promote muscle damage and repair, respectively. Type 1 inflammation is characterized by the increased expression of IFNγ, and is counter-regulated by type 2 inflammatory cytokines such as IL-4 and IL-13 and the anti-inflammatory cytokine, IL-10. Although several studies have elucidated how these inflammatory responses mediate injury and repair ([17–21]), it is not clear how the balance between type 1 and type 2 inflammatory responses is regulated in muscle, or what regulatory role lymphocytes exert on this balance.

Regulatory T cells (Tregs) are candidate immunosuppressive lymphocytes that possess the functional capacity to modulate dystrophinopathy by regulating the balance between type 1 and type 2 inflammatory responses. They are a specialized subset of $CD4^+$ T cells whose lineage specification is dependent on the forkhead transcription factor, FoxP3 ([22]). Tregs express high levels of CD25, glucocorticoid induced TNFR-related protein (GITR), cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed cell death-1 (PD-1). Tregs secrete regulatory cytokines such as IL-10 that endow them with the capacity to maintain immune homeostasis and resolve IFNγ-dependent, T helper 1 cell responses ([23]). Previous

studies have reported that Tregs accumulate in dystrophic muscle ([24]) and have shown that FoxP3 mRNA is increased in muscle from osteopontin-deficient mdx mice, suggesting that their numbers or stability are partly regulated by osteopontin ([25]). Burzyn and colleagues recently demonstrated that Tregs, expressing high levels of amphiregulin and IL-10, accumulated during later stages of muscle repair, and that depletion of CD25-expressing cells, including Tregs, in mdx mice exacerbated muscle injury ([26]). However, a specific examination of the functional role of Tregs in muscular dystrophy is still lacking.

In this investigation, we found that Tregs are elevated in human DMD/BMD and mouse mdx muscle and display an activated phenotype. We used a combination of loss-of-function methods that specifically targeted Tregs to examine if Tregs regulate the pathogenesis and progression of muscular dystrophy. Histological and biochemical analysis of dystrophic muscle depleted of Tregs revealed an increase in immune cell infiltration and an enhanced type 1 inflammatory response, which resulted in exacerbated myofiber damage. The suppressive effect of Tregs on dystrophinopathy was further supported by the amelioration of muscle inflammation and injury in mdx mice treated with IL-2c. Collectively, these findings show that Tregs play a critical role in limiting muscle damage during muscular dystrophy by suppressing the development of type 1 inflammatory responses, and demonstrate the potential clinical value of therapeutically targeting Tregs in DMD.

Results

Tregs are elevated in the muscles of muscular dystrophy patients

The examination of muscle biopsies from control subjects and subjects with dystrophinopathy (Table S1) by epifluorescence microscopy showed that Tregs were increased in DMD/BMD muscle biopsies compared to control muscle biopsies (Fig. 1). Necrotic lesions in dystrophic muscle were distinguished by $CD3^+$ T cell aggregates (green, Fig. 1D) containing $FoxP3^+$ cells (red, Fig. 1E) that were all $CD3^+$ (Fig. 1F); these features were largely absent in controls (Fig. 1A–C). Quantification of lymphoid populations in DMD/BMD and control groups revealed a significant increase in the number of $CD3^+$ ($P < 0.05$) and $FoxP3^+$ ($P < 0.01$) cells in dystrophic muscle, but the frequency of $FoxP3^+$ cells among $CD3^+$ cells was not altered (Fig. 1G–I, and Table S2). Moreover, elevated numbers of $FoxP3^+$ cells coincided with increased IL-10 expression in DMD/BMD muscle compared to control muscle ($P < 0.05$) (Fig. 1J).

Muscle inflammation in mdx mice is associated with increased Tregs

Studies performed on the mdx mouse have shown that inflammation is a key pathological component contributing to the pathogenesis of muscular dystrophy. Thus, we used the mdx mouse model to study the capacity of Tregs to regulate muscle inflammation and injury during muscular dystrophy. Flow cytometry provided a sensitive and quantitative method to determine the cellular composition of the inflammatory infiltrate in mdx muscle (Fig. S1A). We found that the total cellularity obtained from single-cell preparations of collagenase-digested muscle was increased in mdx mice relative to wildtype mice (Fig. S1B), likely attributable to the increased proportion (Fig. S1C) and absolute quantity (Fig. S1D) of $CD45^+$ cells in mdx muscle. Moreover, the proportion of $Thy1^+$ cells was reduced at 4

weeks of age but higher at 12 weeks of age relative to age-matched controls (Fig. S1E). The increased proportion of $Thy1^+$ cells in mdx mice is likely attributable to the reduced numbers of myeloid cells in muscle of 12-week-old mdx mice relative to 4-week-old mice and the relative increase in $CD4^+$ T cells during this time ([11], [27]). The absolute number of $Thy1^+$ cells was increased in mdx mice at all ages examined relative to age-matched controls (Fig. S1F).

Next, we examined the composition of $CD4^+$ T cells and found that the proportion of $CD45^+Thy1^+$ cells in mdx mice was increased at 12 weeks compared to age-matched controls and 4-week-old mdx mice, but did not differ between 4-week-old mdx and wildtype mice (Fig. 2A and 2B). However, the absolute number of $CD4^+$ T cells was increased in mdx mice at all ages examined relative to age-matched controls (Fig. 2C). An extremely rare population of $FoxP3^+$ Tregs was found in wildtype mouse muscle (Fig. 2D–F). However, a substantial increase in the proportion (Fig. 2E) and absolute number (Fig. 2F) of Tregs was found in mdx mouse muscle at 4 and 12 weeks of age. In addition to splenomegaly and increased lymph node cellularity, we found that total $CD4^+$ T cells and $FoxP3^+$ Tregs were also elevated in the para-aortic lymph node (PaLN) of mdx mice (Fig. S2).

Tregs are localized in muscle lesions

Next, we used histological techniques to examine the localization of Tregs in muscle to gain insights into the functional significance of these cells in the pathogenesis of muscular dystrophy. Tregs in wildtype muscle were so rare that they were not detected by immunohistochemical staining of frozen cross-sections of wildtype quadriceps (Fig. 2H) or diaphragm (Fig. S3B). In contrast, $FoxP3^+$ cells were found within inflamed, necrotic lesions in mdx quadriceps (black arrows in Fig. 2J) and diaphragm (Fig. S3D). Muscle sections in which the anti-FoxP3 antibody was omitted during the staining procedure served as negative controls (Fig. 2G, 2I; Fig. S3A and S3C).

Muscle Tregs display an activated phenotype and express IL-10

Representative contour plots show that the frequencies of muscle Tregs expressing the activation markers, killer cell lectin-like receptor subfamily G member 1 (KLGR1) and GITR or PD-1 were increased in mdx mouse muscle relative to wildtype muscle (Fig. 3A). Infrequent populations of $KLGR1^+GITR^+$ and $KLRG1^+PD\text{-}1^+$ Tregs were found in PaLN and were slightly increased in mdx mice at 4 weeks of age relative to age-matched control mice (Fig. 3B, C). However, the frequencies of $KLGR1^+GITR^+$ and $KLRG1^+PD\text{-}1^+$ muscle Tregs were greatly increased relative to PaLN, with a greater increase in mdx muscle relative to wildtype muscle (Fig. 3D, $P < 0.05$; 3E, $P < 0.01$). Although PD-1 was not significantly increased, the expression of KLRG1 and GITR was increased in mdx muscle Tregs (Fig. 3F–H). Interestingly, KLGR1 was not expressed on muscle conventional T cells (Tconv) (Fig. S4); thus, we examined the frequencies of single-positive GITR and PD-1 muscle Tconv. We found that $GITR^+$ Tconv were elevated in mdx mice at all ages examined (Fig. S4B) and $PD\text{-}1^+$ Tconv were elevated at 12 weeks (Fig. S4C). Although GITR expression on mdx muscle Tconv was increased relative to age-matched controls, the expression was lower than that found on Tregs (Fig. S4D). No difference in the expression of KLRG1 or PD-1 was observed between mdx and wildtype muscle Tconv (Fig. S4E, S4F).

Recently, Tregs accumulating in acutely injured muscle were shown to express high levels of IL-10 ([26]), a suppressive cytokine which was previously shown to reduce the pathology of muscular dystrophy in mdx mice ([20]). RT-qPCR analysis revealed that IL-10 was expressed by multiple immune cell subsets purified from mdx muscle by fluorescence-activated cell sorting (Fig. 3I). Interestingly, IL-10 was not expressed substantially in PaLN Tregs at 4 or 12 weeks of age in mdx mice when a large increase in IL-10 was detected in muscle Tregs (Fig. 3I).

Depletion of Tregs exacerbates muscle inflammation and injury in mdx mice

The increased number, activation and localization of Tregs in dystrophic muscle suggests that they suppress myofiber injury due to the chronic muscle inflammation triggered by the mechanical frailty of dystrophin-deficient myofibers. Mdx mice were treated with a Treg-depleting anti-CD25 antibody, clone PC61, to test this hypothesis ([28]). We began treatment at 3 weeks of age when disease onset occurred to prevent difficulties in interpreting the effect on Treg depletion on dystrophinopathy since PC61 treatment in neonatal mice causes multi-organ autoimmunity. Treated mdx mice were sacrificed at 6 weeks of age to allow sufficient time for the development of muscle injury and remodeling. As seen previously ([29]), PC61 treatment significantly decreased the proportion of $CD4^+CD25^+$ T cells (Fig. S5A) and $FoxP3^+$ Tregs (Fig. S5B) in mdx mice. A histological examination of quadriceps muscle sections revealed an increased inflammatory infiltrate in muscle of mdx mice treated with PC61 relative to control-treated mdx mice (Fig. 4A, 4B). In addition, PC61 treatment increased the expression of IFNγ and TGFβ in mdx muscle (Fig. 4C), suggesting that Tregs or other $CD25^+$-expressing cells regulate the expression of these cytokines in dystrophic muscle. However, PC61 treatment did not influence the expression of Th2, IL-10 or IL-17A cytokines. Importantly, PC61 treatment increased the number of myofibers with accumulated albumin, indicating increased muscle injury following Treg depletion (Fig. 4D–4F).

Given that PC61 may deplete non-Treg cells that express CD25, the altered cytokine expression following PC61 treatment may not be due solely to the depletion of Tregs. Therefore, mdx mice were crossed with the DEREG mouse in which the FoxP3 promoter controls the expression of a GFP-diphtheria toxin receptor (DTR) transgene ([30]). This animal model (mdx^{DEREG}) facilitated the specific and acute ablation of lymph node (Fig. S6A) and muscle Tregs (Fig. S6B) in mdx mice following diphtheria toxin (DT) treatment. Consistent with previous studies ([31]), a rapid recovery of Treg numbers was observed following DT treatment (black line, Fig. S6A and S6B), but a substantial proportion did not express the GFP-DTR fusion protein (grey line vs. dashed line, Fig. S6A and S6B). Thus, the recovering Tregs could not be fully ablated by repeated treatment with DT.

On the 4^{th} day after initiation of DT treatment, muscle (Fig. 4G, $P < 0.01$) and PaLN Tregs (Fig. S6C and S6D, $P < 0.0001$) were still significantly reduced, whereas the frequency of activated Tconv in muscle (Fig. 4H, $P < 0.05$) and PaLN (Fig. S6D, $P < 0.001$) was increased. Similar to PC61-treated mdx mice, we found that IFNγ was significantly increased in mdx^{DEREG} muscle that was depleted of Tregs (Fig. 4I, $P < 0.05$). Although a decrease in IL-5 was also seen, depletion of Tregs did not influence the expression of other

Th2, IL-10 or IL-17A cytokines. To define the cellular source of IFNγ, we sorted bulk populations of myeloid ($CD45^+Thy1^-$) and lymphoid ($CD45^+Thy1^+$) cells and found that IFNγ was predominantly confined to the lymphoid compartment in mdx mice (Fig S6E). Intracellular cytokine staining of $CD4^+$ T cells revealed an increased frequency of muscle Tconv expressing IFNγ (Fig. 4J, 4K) and expression of genes upregulated during a Th1 response, namely granzyme B, PD-1 and CXCR3 (Fig. 4L). The frequency of IL-17A-expressing muscle Tconv was not affected by Treg ablation, suggesting that Tregs specifically suppressed type 1 inflammatory responses during muscular dystrophy (Fig. S6F).

Tregs regulate macrophage activation in muscle

In addition to promoting M1 activation of proinflammatory macrophages that contribute to muscle injury ([19]), IFNγ also induces the expression of PD-L1 ([32]). Thus, PD-L1 serves as a sensitive readout of IFNγ-mediated M1 macrophage activation. We found discrete populations of M1 ($CD11b^+F480^+SiglecF^-Ly6c^+CD301^-$) and M2 ($CD11b^+F480^+SiglecF^-Ly6c^-CD301^+$) macrophages in muscle of mdx mice that harbored a DTR transgene at the 3′ end of the endogenous FoxP3 locus ($mdx^{FoxP3.DTR-KI}$) (Fig. 5A). $Mdx^{FoxP3.DTR-KI}$ mice allowed for the chronic depletion of Tregs with repeated DT treatment without the rebound of Tregs observed in mdx^{DEREG} mice (Fig. S7). The depletion of Tregs in $mdx^{FoxP3.DTR-KI}$ mice (~90% reduction) resulted indirectly in a significant increased expression of PD-L1 on M1 ($P < 0.05$)and M2 ($P < 0.001$) macrophages without affecting the proportion of either population in dystrophic muscle following an 11 day depletion period (Fig. 5B, 5D and 5E). Moreover, Treg depletion significantly ($P < 0.05$) decreased the expression of the M2 activation marker, CD206, on M2 macrophages but had no effect on M1 macrophages (Fig. 5C, 5F and 5G).

Therapeutic targeting of Tregs reduces muscle inflammation and injury in dystrophic mice

To assess the value of targeting Tregs as a potential treatment for DMD, we treated mdx mice with low-dose IL-2c that selectively increased Tregs in vivo ([33]). We found that IL-2c significantly ($P < 0.05$) increased the frequency of $FoxP3^+$ Tregs (Fig. 6A and 6B) and the expression of IL-10 (Fig. 6C) in hind limb muscles of mdx mice. We next utilized an $mdx.cox\text{-}2^{Luc/+}$ reporter mouse to assess the effect of IL-2c treatment on muscle inflammation. Although no difference in luciferase signal was detected at 4 and 5 weeks of age, a significant ($P < 0.05$) decrease in signal intensity was observed in exercised 6-week-old mdx mice treated with IL-2c (Fig 6D). Representative heat map images of mice used for non-invasive imaging revealed a reduced luciferase signal in the hind limb muscles of $mdx.cox\text{-}2^{Luc/+}$ mice treated with IL-2c, indicating that IL-2c treatment reduced muscle inflammation (Fig. 6E). Importantly, the reduced inflammation in IL-2c-treated mice was accompanied by a decrease in serum creatine kinase concentrations, a serum biomarker of muscle injury (Fig. 6F). These results were further corroborated by the histological observation that IL-2c treatment decreased the proportion of myofibers in the diaphragm that incorporated serum albumin, indicating reduced muscle injury (Fig. 6G and 6H).

Discussion

Our findings suggest that Tregs control the progression and pathophysiology of muscular dystrophy by restraining the development of a type 1 inflammatory response in muscle. We found that Tregs were elevated in muscle of human muscular dystrophy patients and mdx mice but largely absent in non-injured muscle, suggesting that downstream events following muscle injury are contributing factors that drive Treg accumulation in dystrophic muscle. The localization of Tregs in necrotic lesions and their state of activation suggest that they actively suppress muscle inflammation. Indeed, we found that depletion of Tregs in mdx mice enhanced immune cell infiltration, muscle injury, and increased the expression of IFNγ by muscle Tconv in mdx mice.

Previous investigations have shown that IFNγ is chronically overexpressed in dystrophic muscle, increasing the M1:M2 macrophage ratio to promote myofiber injury ([18], [19]). Moreover, the genetic deletion of IFNγ in mdx mice reduces the severity of muscular dystrophy, further supporting a role for IFNγ in the pathogenesis of muscular dystrophy ([18]). Our present findings that $Thy1^+$ lymphocytes, including $CD4^+$ effector T cells, are the predominant source of IFNγ in dystrophic muscle, suggest that the chronic expression of IFNγ is due to the expansion of these effector T cells and not myeloid cells such as macrophages. Importantly, our findings also indicate that muscle Tregs act centrally at the interface of muscle immunity and immune regulation by restraining the production of IFNγ by $CD4^+$ effector T cells. Although we propose the increased expression of IFNγ following Treg depletion is responsible for the enhanced M1 activation of macrophages, we cannot rule out the possibility that regulatory mechanisms that directly act on macrophages to suppress M1 activation are also lost. Indeed, we found that the depletion of Tregs decreased IL-5, a Th2 cytokine that promotes the generation of eosinophils that are critical for the development of type 2 inflammation in injured muscle ([17]). However, whether the Treg-mediated regulation of IL-5 has any effect on muscle eosinophilia or whether the type 2 inflammatory responses mediated by eosinophils contribute to M2 activation of macrophages in mdx mice remains to be determined.

Tregs control immune homeostasis and restrain inflammatory responses through a diverse number of molecular pathways, including the expression of the immune regulatory cytokine, IL-10. For instance, during experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis in which disease is cooperatively driven by Th17 and Th1 immunity, Tregs suppressed the severity of EAE by inhibiting the expression of IL-17 and IFNγ in an IL-10-dependent manner ([34]). Importantly, transcriptional profiling of muscle Tregs isolated from acutely-injured muscle revealed that muscle Tregs express exceptionally high levels of IL-10 compared to other lymphoid organ Tregs ([26]). Similarly, our findings that mdx muscle Tregs are a rich source of IL-10 suggest that the suppression of type 1 muscle inflammation by Tregs is IL-10-dependent. In support of this model, ablation of IL-10 in mdx mice has been shown to increase the severity of muscular dystrophy ([20]). Furthermore, in addition to increasing muscle Tregs and reducing muscle injury and inflammation, IL-2c treatment also increased the expression of IL-10 in mdx mouse muscle. However, an acute or partial depletion of Tregs in our study failed to show a change in IL-10 expression, suggesting that compensatory mechanisms might be at play or that additional regulatory mechanisms

cooperate with IL-10 for the Treg-mediated suppression of type 1 inflammation in muscle. Studies using IL-10 conditional knockout mdx mice in which IL-10 is specifically ablated in Tregs will aid in revealing the functional role of Treg-derived IL-10 in the pathogenesis of muscular dystrophy.

In addition to their control of chronic inflammation, Tregs are critical in the maintenance of tolerance to self-antigens, as evidenced by catastrophic autoimmunity in mice and humans that lack FoxP3 ([35–37]). Although known auto-antigens that drive muscle antigen-specific immune responses remain ill-defined, there is evidence that clonal expansion of auto-reactive T cells in mdx mice and DMD patients does occur. In studies examining the pathogenic role of osteopontin in mdx mice, investigators found an enrichment of Vβ8.1/8.2$^+$ T cells in muscle ([25]), suggesting an oligoclonal expansion of T cells in response to presentation of muscle antigens following injury. In humans, sequencing of the CDR3 region of the TCR Vβ2 chain from DMD muscle biopsy revealed a conserved rearrangement, indicating that a common antigen within these patients was also driving a muscle antigen-specific immune response ([38]). Our observation that muscle CD4$^+$ T cells are activated in mdx mouse muscle is in agreement with the hypothesis that muscle antigens released after injury promote the activation and effector function of muscle T cells. In this regard, Tregs are likely to prevent muscle autoimmunity and restrain inflammation during the course of injury and antigen release.

In addition to self-antigens that promote immunity, the concern about autoreactive T cell responses also extends to dystrophin gene therapy, which introduces a neo-antigen that may be immunogenic. A recent study showed that a sizable proportion of DMD patients have a preexisting pool of dystrophin reactive T cells ([39]). Moreover, a recent dystrophin gene therapy trial performed in a small number of patients revealed that a subset developed dystrophin-specific T cell responses ([40]). These preliminary observations together with the concern of unwanted side effects associated with chronic glucocorticoid therapy has prompted recent interest in developing immunotherapies that not only mitigate inflammation but also suppress dystrophin immunity ([41]). In this regard, our data implicate Tregs or Treg-boosting agents as potential therapies that may be used in conjunction with gene therapy as an alternative approach to limit dystrophin immunity while limiting side effects associated with glucocorticoids. Further studies will be required to fully address the significance of the generation of dystrophin immunity during gene therapy, along with studies aimed at examining the capacity of Tregs to suppress dystrophin immunity during gene therapy.

The molecular attributes of muscle Tregs suggest that their function extends beyond mere regulators of muscle immunity to modulators of tissue repair and remodeling processes. In fact, Burzyn et al. recently reported that muscle Tregs express amphiregulin, and that treatment of injured muscle with exogenous amphiregulin improved muscle regeneration, possibly by promoting satellite cell differentiation ([26]). Moreover, they showed that the depletion of Tregs during acute muscle injury did not only impair muscle healing but also increased fibrosis. Interestingly, we found that chronic depletion of Tregs with PC61 treatment increased the levels of the pro-fibrotic factor, TGFβ, further supporting a relationship between Tregs and the negative regulation of fibrosis. However, it remains to be defined whether amphiregulin negatively regulates the expression of TGFβ or whether other

factors are responsible for this regulation. In this regard, recent studies have shown that osteopontin deficiency in mdx mice increased FoxP3 mRNA in muscle, but reduced TGFβ and muscle fibrosis ([25]). Moreover, we have found that Tregs make an anti-fibrotic factor, relaxin-3 ([42]), that is overexpressed in mdx mice as Treg numbers increase in mdx muscle. Considering that relaxin-3 also has anti-inflammatory properties, it is interesting to speculate that in addition to reducing fibrosis, relaxin-3 may suppress muscle inflammation independently or cooperatively with IL-10. It is important to note that the depletion of Tregs in the mdx^{DEREG} mouse did not increase TGFβ, which may be explained by the acute nature of the Treg depletion in this model. Alternatively, the increased TGFβ and decreased IL-5 in PC61-treated mice might be attributed to the depletion of a non-Treg cell population that expresses CD25.

Collectively, our data and previously reported findings support a model in which as yet unidentified signals arising from muscle injury recruit and expand muscle Tregs that restrain type 1 muscle inflammation and secrete factors that enhance muscle regeneration. Specifically, Tregs suppress the expression of IFNγ by $CD4^+$ T cells and the subsequent activation of muscle injury-promoting M1 macrophages. IL-10 is a candidate molecule that may mediate the suppressive function of muscle Tregs during muscular dystrophy, but the lack of change in IL-10 following Treg depletion suggests that alternative mechanisms are also involved. Because IFNγ can directly inhibit myoblast proliferation ([43]), Tregs also participate in muscle regeneration by inhibiting the anti-myogenic activity of IFNγ expressed by Tconv, and through the secretion of factors, such as amphiregulin, that act directly on myogenic cells. Considering that fibrosis is a prominent feature of DMD that is exacerbated by Treg depletion, further research is warranted to understand the relationship between Tregs and the development of fibrosis during muscular dystrophy, and how this axis cooperates with amphiregulin, or other regenerative factors (i.e. relaxin-3), to promote muscle regeneration. As mentioned above, human DMD patients and mdx mice, although sharing many pathological features, differ in the rate of disease progression and the severity of muscle deterioration and loss of muscle function. It is interesting to speculate that such variations in clinical presentation may be due to differences in the phenotypic character, functional state or altered recruitment kinetics of muscle Tregs in human versus mouse. In summary, our studies and those reported recently by other investigators ([26]) reveal the therapeutic potential of targeting Tregs in muscular dystrophy and other muscle disorders.

Materials and Methods

Study design (human)

We aimed to study the number and function of immune cells within the tissue of humans with DMD and BMD. To identify dystrophinopathy samples for histologic studies, we performed a computerized search of the UCSF neuropathology case database spanning the interval between 2002 and 2013; 5 muscle biopsies for which frozen tissue was available were included in the dystrophinopathy group. Two additional samples were analyzed by collaborators at UCLA (Dr. Spencer). For the control group, patients were biopsied because of clinical suspicion for some muscle disease (i.e. necrotizing myopathy, inflammatory myopathy), but blinded sampling yielded uninvolved/normal tissue. Group assignment was

based on histologic features of dystrophinopathy alone and no attempt was made to match participants by age, sex, or other demographic variables (Table S1). Immunohistochemical and immunofluorescent staining of frozen cross-sections was performed as previously described ([19]). Anti-human FoxP3 antibody (eBioscience) was applied to muscle sections overnight at 4°C. Alexa-conjugated secondary antibodies were used to visualize primary antibody-specific staining. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Bright field and epifluorescent images were captured with a Zeiss AxioImager microscope or Zeiss Apotome microscope, respectively. The morphological features of muscular dystrophy were assessed by staining formalin-fixed cross-sections of mdx muscle with hematoxylin. Representative images of H&E-stained muscle sections showing the severity of muscle pathology in patients with muscular dystrophy and controls are provided (Fig. S8). The study design was reviewed and approved by the University of California San Francisco (UCSF) Committee on Human Research (CHR). For studies on archival muscle biopsy tissue, the informed consent requirement was waived by the CHR given a minimal potential for harm to study participants. No individually identifiable patient data is presented.

We aimed to study the number and function of immune cells within the tissue of mice with muscular dystrophy as well as study the functional consequence of Treg manipulation. Thus, we set up a number of animal studies designed to evaluate Tregs in muscle tissues and study inflammation during disease progression in mdx versus control mice. To accomplish this, C57BL/6, C57BL/10, and B6.129(Cg)-FoxP3$^{tm3(DTR/GDP)Ayr}$/J mice (purchased from The Jackson Laboratory, Bar Harbor, ME) and DEREG mice ([30]), provided by Tim Sparwasser were crossed with mdx mice (C57BL/10ScSn-Dmd^{mdx}/J, Jackson Laboratory). Mdx.cox2$^{Luc/+}$ were created by crossing the mdx mouse with the cyclooxegenase2 (Cox2)-luciferase knock-in mouse created by Dr. Harvey Herschman, which was backcrossed a minimum of 5 times to C57BL/6 background ([44]). F1 Heterozygous littermates were intercrossed to obtain luciferase/cox2 heterozygous and dystrophin-null mice (mdx.cox2$^{Luc/+}$). The cox-2 luciferase knock-in allele was always maintained in the heterozygous state. All mice were bred in a pathogen-free facility at the University of California, San Francisco and animal experiments were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco or the University of California, Los Angeles.

Mouse histology

Immunohistochemical and immunofluorescent staining of frozen cross-sections was performed as previously described ([19]). Briefly, anti-mouse FoxP3 (eBioscience) or anti-human serum albumin antibody (AbD Serotec) was applied to muscle sections overnight at 4°C. Mouse FoxP3 staining was visualized with secondary immunoreagents and 3-amino-9-ethylcarbazole peroxidase substrate (Vector Laboratories). Alexa-conjugated secondary antibodies were used to visualize primary antibody-specific staining. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Bright field and epifluorescent images were captured with a Zeiss AxioImager microscope or Zeiss Apotome microscope, respectively. The morphological features of muscular dystrophy were assessed by staining formalin-fixed cross-sections of mdx muscle with hematoxylin.

In vivo Treg ablation

CD25^{+} Tregs were depleted in mdx mice using a modification of an in vivo depletion protocol previously described ([29]). 3-week-old mdx mice were injected twice with 250 μg of an anti-CD25 antibody (clone PC61). Mice were sacrificed and muscles harvested at 6 weeks of age for histological examination to assess the effect of Treg depletion on muscular dystrophy. To specially deplete Tregs, mdx mice were crossed to the DEREG mouse or the FoxP3.DTR-GFP knock-in (FoxP3.DTR-KI) mouse. Tregs in 4–5-week-old mdx$^{\text{DEREG}}$ mice were depleted with two consecutive daily intraperitoneally (IP) injections of diphtheria toxin (40 μg/kg) and sacrificed at day 4. Tregs were depleted in 3-week-old mdx$^{\text{FoxP3.DTR-KI}}$ by injecting DT every other day over an 11 day period. No mortalities or signs of overt morbidity (i.e. dehydration, lethargy or ruffled coat) were observed in mdx$^{\text{FoxP3.DTR-KI}}$ in the time frame and dosage in these experiments. A small loss of body mass was detected as reported previously ([45]).

Muscle injury assays

Injured fibers that allow the diffusion and accumulation of serum proteins in their cytoplasm were identified in cross-sections of quadriceps muscles by immunostaining with anti-albumin (AbD Serotec) followed by an Alexa 555-conjugated secondary antibody. Imaging was performed on a Zeiss Apotome microscope. The total and injured muscle area was measured with MetaMorph automation and image analysis software (Molecular Devices).

IL-2c treatment of mdx mice

The treatment of mdx mice with IL-2/anti-IL-2 complexes was initiated at 2 weeks of age. 3 doses were administered IP every other day during the first week of treatment. Each dose consisted of 0.5 μg of recombinant mouse IL-2 (eBioscience) pre-incubated with 5.0 μg of anti-IL-2 antibody (clone JES6-1A12, R&D Systems) for 15 minutes at 37°C. Mice were sacrificed at 6 weeks of age and muscles and serum samples were collected for analysis.

In vivo bioluminescent imaging

To assess inflammation non-invasively, optical imaging (IVIS, Xenogen) was used to assess bioluminescent activity in IL-2c treated and untreated mdx.cox-2$^{\text{Luc/+}}$ mice, based on the observation that immune cells express cox-2 upon activation. Mice were imaged at 4, 5, and 6 weeks of age. Because the luciferase signal in mdx.cox-2$^{\text{Luc/+}}$ mice is below the level of detection in rested animals, mice were exercised on a downhill treadmill at 19 m/m for 20 minutes, and then imaged 2 days post exercise were luciferase activity was then detected. For imaging, mice were weighed, anesthetized with 2% isoflorane, limbs shaved and injected IP with D-luciferin (125mg/kg). Mice were placed in a temperature-controlled chamber, ventral side up, within the IVIS imaging unit, and images were acquired with one-minute scans using medium binning at 15, 20, 25, and 30-minute time points. Living Image®4.0 software was used for the post-acquisition analysis of bioluminescent activity measured in photons and expressed in Max Radiance. Bioluminescent measurements of the hind limbs were averaged together for each animal for statistical analysis.

Serum creatine kinase

To assess muscle membrane integrity, serum creatine kinase levels (CK) was assessed using a Creatine Kinase kit (Sekisui Diagnostics P.E.I. Inc.). Blood was collected via retro-orbital bleeding. Serum was isolated and immediately frozen at −80°C.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5.01. Statistical comparisons between two groups were performed using an unpaired two-tailed Student *t* test or a nonparametric Mann-Whitney test. Comparisons between multiples groups were performed by one-way or two-way ANOVA, followed by a post hoc Bonferroni test to determine significance of differences between two groups. Values of $P \leq 0.05$ were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Accessible Summary

Duchenne Muscular Dystrophy (DMD) is caused by dystrophin gene mutations leading to muscle degeneration. We show that the imbalance between pro-inflammatory and pro-regulatory immune cells in both humans with DMD and MDX mice is directly involved in myofiber damage. Altering anti-inflammatory Regulatory T cells (Treg) controls tissue destruction and clinical manifestations. Treg elimination results in increased production of pro-inflammatory cytokines and macrophages while treatment with a Treg-promoting cytokine, interleukin-2, increased immunosuppressive interleukin-10 production and resolved myofiber damage. Thus, Tregs modulate the progression of muscular dystrophy by suppressing type 1 inflammation and highlight the potential of Treg-modulating agents as DMD therapeutics.

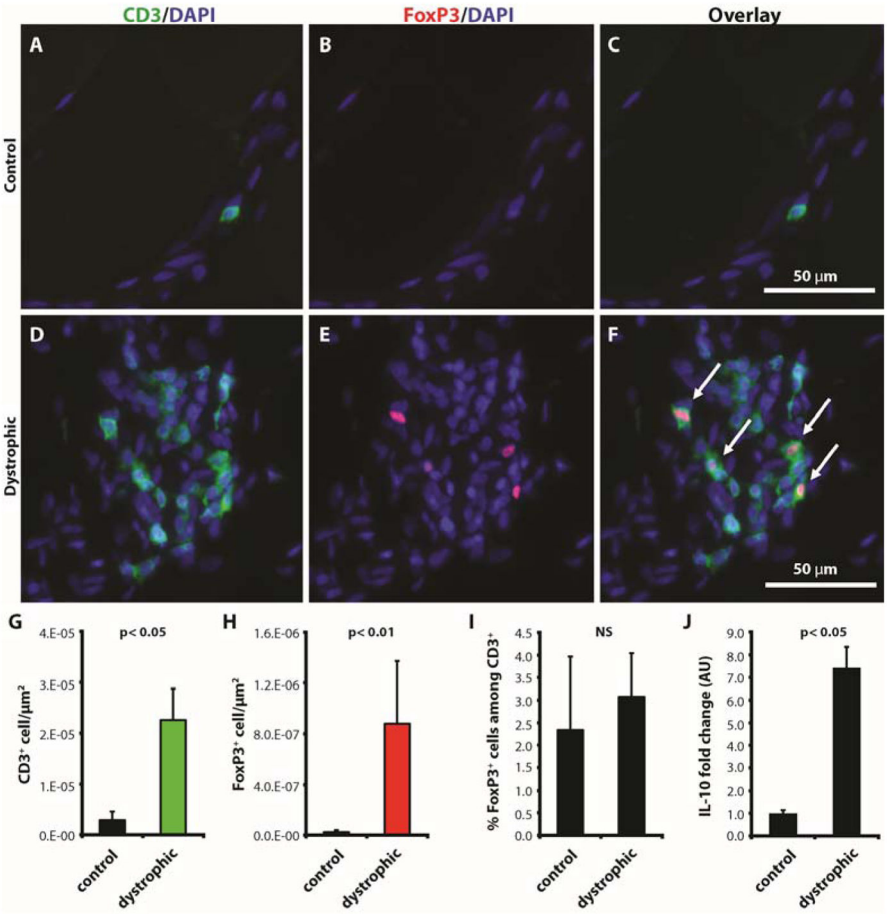

Figure 1. Tregs are elevated in muscle of human subjects with DMD/BMD

(**A–F**) Immunofluorescence staining of CD3 (green, A and D) and FoxP3 (red, B and E) in control (AC) and DMD (D–F) muscle sections. Overlaid images reveal that FoxP3$^+$ cells express CD3 (F, white arrows); nuclei were counterstained with DAPI (blue). Quantification of the number of CD3$^+$ (**G**) and FoxP3$^+$ (**H**) cells per tissue sectional area. (**I**) The frequency of FoxP3$^+$ cells among CD3$^+$ cells. (**J**) IL-10 mRNA in whole muscle was measured by RT-qPCR. 5 and 7 muscle samples were analyzed for the control and dystrophic groups, respectively. Statistical significance was determined by the nonparametric Mann-Whitney test.

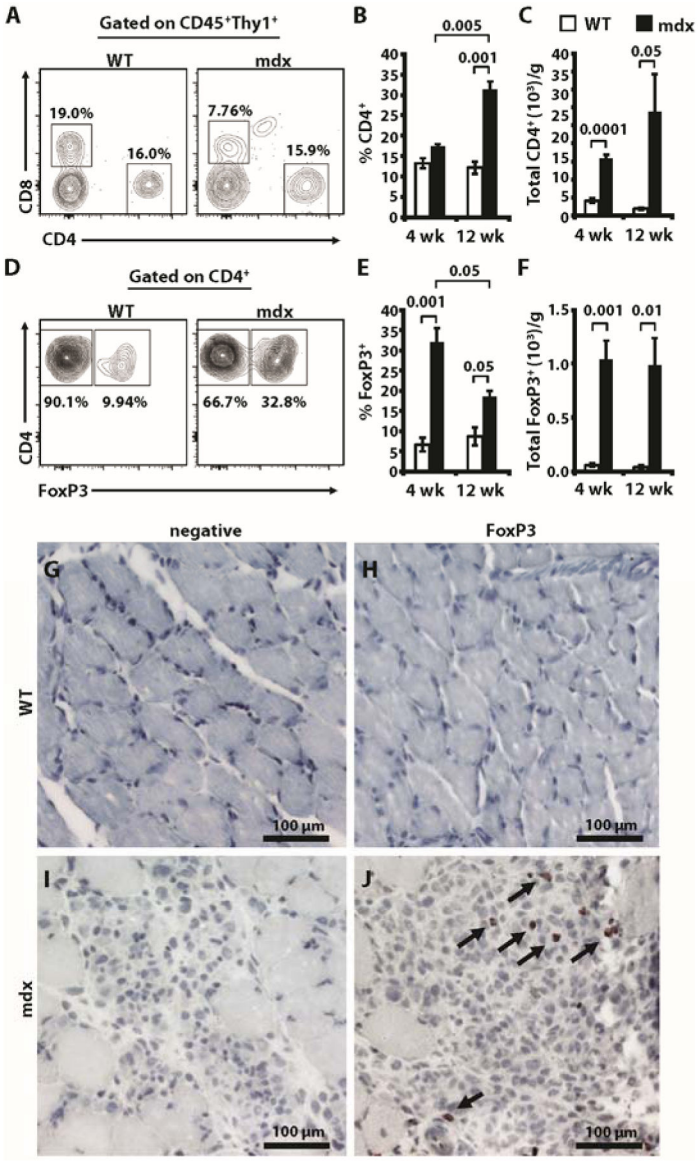

Figure 2. T regs are elevated in mdx mice

(**A**) Representative contour plots showing the frequency of $CD4^+$, $CD8^+$ and $FoxP3^+$ T cells in muscle. (**B–C**) The frequency (B) and absolute number (C) of $CD4^+$ T cells normalized to muscle mass. (**D–F**) The frequency (E) and absolute number (F) of $FoxP3^+$ cells in wildtype (WT) and mdx muscle. The data are presented as a cumulative analysis of 6 independent experiments with 4–5 mice per group. The numerical values in B, C, E and F reflect the level of statistical significance determined by a two-tailed *t* test. (**G–J**) 4-week-old WT (G and H) and mdx (I and J) muscles showing the distribution of $FoxP3^+$ cells. Shown are negative controls in which the FoxP3 antibody was omitted (G and I) and sections stained with anti-FoxP3 antibody (H and J). Black arrows in J highlight $FoxP3^+$ cells that reside in necrotic/inflammatory lesions in muscle.

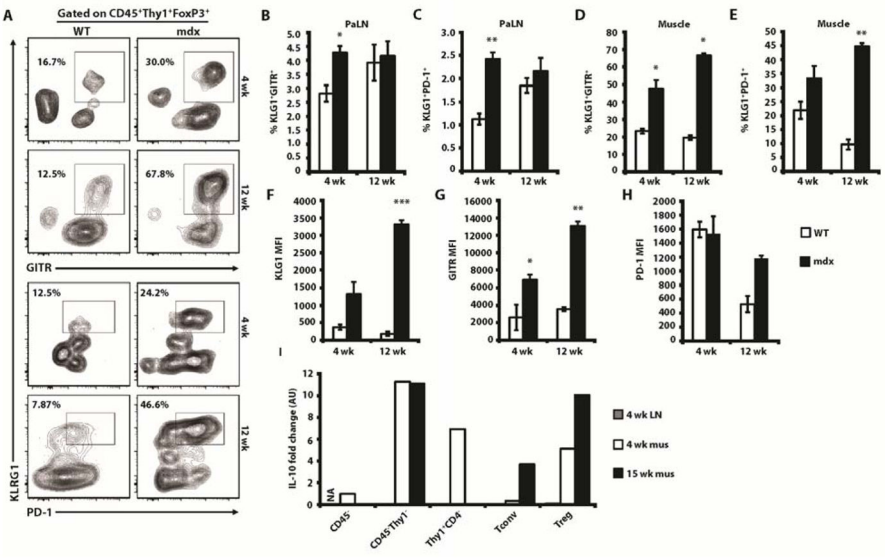

Figure 3. Tregs are activated in dystrophic muscle

(**A–E**) Representative contour plots showing the frequency of KLRG1^{+}GITR^{+} and KLRG1^{+}PD-1^{+} cells among viable CD45^{+}Thy1^{+}CD4^{+}FoxP3^{+} Tregs in wildtype (WT) and dystrophic mdx muscle (A) and their quantification (B–E) in PaLN (B and C) and mdx muscle (D and E). (**F–H**) Quantification of the expression (median fluorescence intensity, MFI) of KLRG1 (F), GITR (G) and PD-1 (H) in muscle Tregs. Data reflect a cumulative analysis of 7 independent experiments. 4-week-old WT mice; n=3, 5–7 mice pooled per sample. 4-week-old mdx mice; n= 8; 1 mouse per sample. 12-week-old WT mice; n=2, 3–4 mice per sample. 12-week-old mdx mice; n= 3, 1 mouse per sample. (**I**) Quantification of IL-10 expression by RT-qPCR in muscle cell populations sorted from 4 and 15-week-old mdx$^{\text{DEREG}}$ mice. Data reflect a cumulative analysis of two independent experiments performed at 4 and 15 weeks of age. 5–6 mice were pooled into one sample for each population and age analyzed. LN= pooled lymph nodes; mus= hind limb muscles pooled from 5–6 mice. Tconv= CD45^{+}Thy1^{+}CD4^{+}FoxP3(GFP)$^{-}$ T cells; Treg= CD45^{+}Thy1^{+}CD4^{+}FoxP3(GFP)$^{+}$ T cells. The levels of statistical significance determined by *t* test with Welch's correction are defined as:*, P< 0.05; **= p< 0.01; ***= p< 0.001.

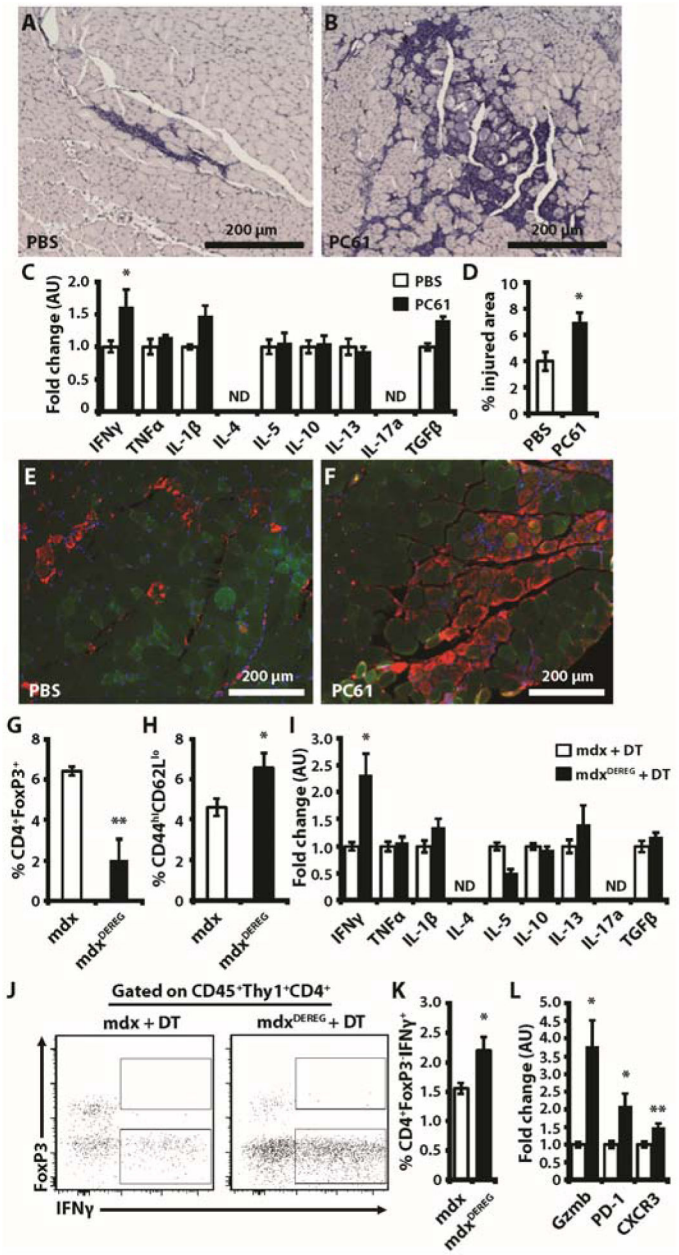

Figure 4. Treg depletion increases the IFNγ response in muscle and exacerbates muscular dystrophy

(**A–B**) Hematoxylin-stained quadriceps muscle from 6-week-old control (A) and PC61-treated (B) mice. (**C**) The expression of inflammatory cytokines in control- and PC61-treated mdx muscle. (**D**) Muscle injury was quantified and expressed as the percent albumin-positive muscle area over total muscle area. 5–8 mice were used per group. (**E–F**) Representative images of quadriceps muscle from PC61-treated mdx mice (F) and control mice (E). Red, green and blue staining reveals albumin-positive muscle, total muscle and nuclei, respectively. (**G–L**) The effect of Treg depletion on muscle inflammation in mdx^{DEREG} mice. 5–7 mice were used per group. The frequency of CD4^{+}FoxP3^{+} Tregs gated

on $CD45^+Thy1^+$ (G) and of $CD44^{hi}CD62L^{lo}$ T cells gated on $CD45^+Thy1^+CD4^+Foxp3^-$ (H). The expression of inflammatory cytokines was measured in whole muscle by RT-qPCR (I) and IFNγ intracellular staining of muscle $CD45^+Thy1^-CD4^+$ T cells (J). Shown are the frequency of muscle $CD4^+FoxP3^-IFN\gamma^+$ effector T cells (K). n= 4–5 per group; each sample represents the pooling of cells isolated from two mice. The expression of effector molecules associated with a Th1 response measured by RT-qPCR (L). n= 7–9 per group. Statistical significance determined by *t* test is defined as: *= $p< 0.05$; **= $p< 0.01$. Data are a cumulative analysis performed on 2–3 independent experiments.

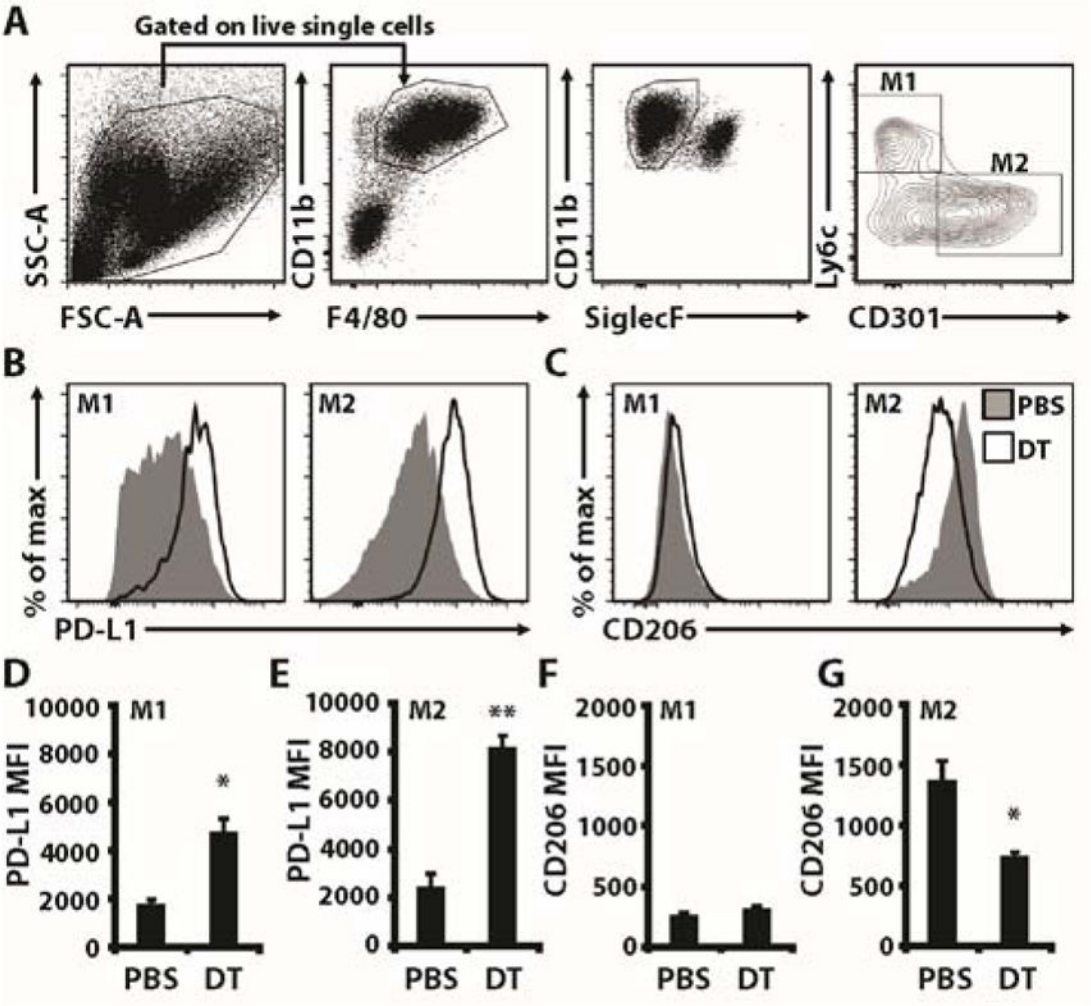

Fig 5. Tregs regulate macrophage activation

(**A**) Representative dot plots showing the gating strategy used to define M1 and M2 macrophage populations. (**B, C**) Representative histograms of PD-L1 (B) and CD206 (C) expression by macrophages in muscle. (**D–G**) Quantification of the MFI of PD-L1 (D and E) and CD206 (F and G) expression by M1 (D and F) and M2 (E and G) macrophages. Statistical significanceis defined as: *= $p < 0.05$; **= $p < 0.01$ [*t* test with Welch's correction]. Data are representative of two independent experiments; n= 3 per group.

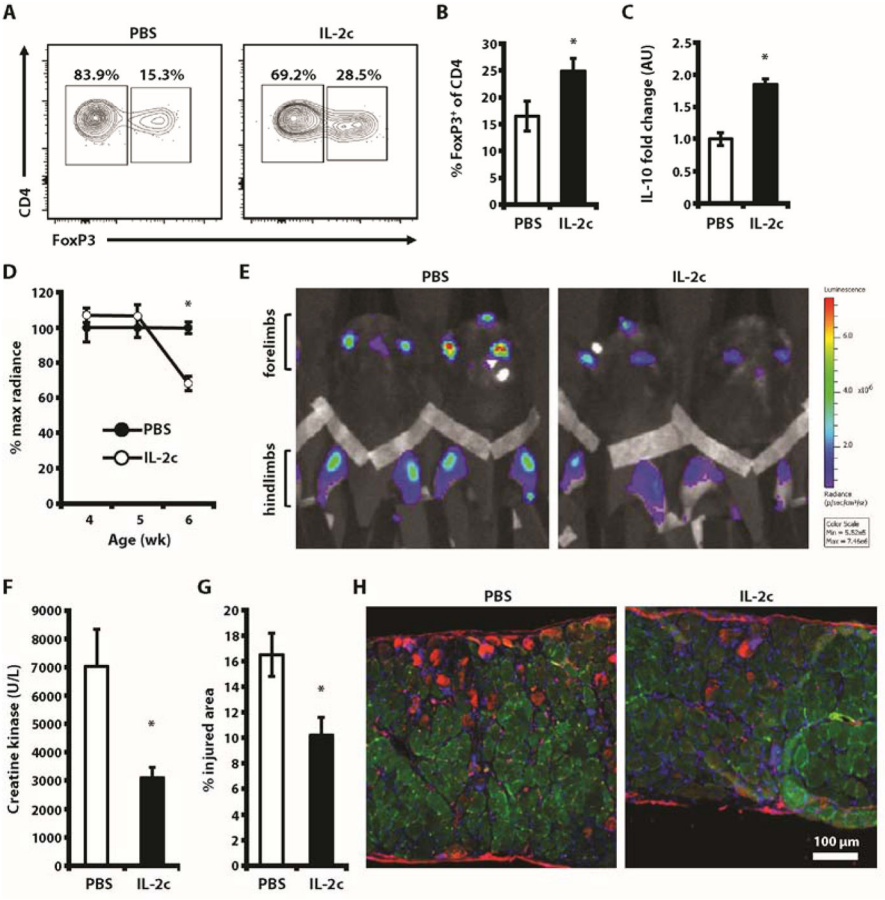

Figure 6. IL-2 complexes augment muscle Tregs in vivo and ameliorate muscular dystrophy

(**A**) Representative contour plots showing the proportion of FoxP3^{+} Tregs (gated on viable, SSC^{lo}FSC^{lo}CD45^{+}Thy1^{+}CD4^{+} T cells) in IL-2c-treated- and control mdx mouse muscle. (**B**) The frequency of muscle Tregs in mdx mice treated with IL-2c. (**C**) Expression of IL-10 mRNA in quadriceps muscle of control and IL-2c-treated mdx mice. Data are representative of 2 independent experiments. (**D**) Quantification of the % max radiance over time following IL-2c treatment. (**E**) Representative whole-body images of IL-2c-treated and control mdx.cox2$^{Luc/+}$ mice, showing pseudocolor IVIS bioluminescence signatures over shaved forelimbs and hindlimbs. (**F**) Serum creatine kinase activity in IL-2c-treated and control mice. (**G**) The injured muscle area of IL-2c-treated and control mice expressed as a percentage of the total muscle area. (**H**) Representative muscle sections showing albumin-positive injured myofibers (red) and the total muscle area (green). Nuclei were counter-stained with DAPI (blue). The data are representative of 4–7 mice per group performed over 3 independent experiments. Statistical significance is defined as *P< 0.05 [*t* test].