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Review

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Review

The potential for Treg-enhancing therapies in tissue, in particular skeletal muscle, regeneration

Bola S. Hanna[1,†], Omar K. Yaghi[1,†], P. Kent Langston[1,†] and Diane Mathis[1,*]

[1]Department of Immunology, Harvard Medical School and Evergrande Center for Immunologic Diseases, Harvard Medical School and Brigham and Women's Hospital; Boston, USA

[†]These authors contributed equally.
*Correspondence: Diane Mathis, Department of Immunology, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, MA 02115, USA. Email: dm@hms.harvard.edu

Summary

Foxp3$^+$CD4$^+$ regulatory T cells (Tregs) are famous for their role in maintaining immunological tolerance. With their distinct transcriptomes, growth-factor dependencies and T-cell receptor (TCR) repertoires, Tregs in nonlymphoid tissues, termed "tissue-Tregs," also perform a variety of functions to help assure tissue homeostasis. For example, they are important for tissue repair and regeneration after various types of injury, both acute and chronic. They exert this influence by controlling both the inflammatory tenor and the dynamics of the parenchymal progenitor-cell pool in injured tissues, thereby promoting efficient repair and limiting fibrosis. Thus, tissue-Tregs are seemingly attractive targets for immunotherapy in the context of tissue regeneration, offering several advantages over existing therapies. Using skeletal muscle as a model system, we discuss the existing literature on Tregs' role in tissue regeneration in acute and chronic injuries, and various approaches for their therapeutic modulation in such contexts, including exercise as a natural Treg modulator.

Keywords: Tregs, tissue regeneration, tissue repair, skeletal muscle, muscular dystrophy, exercise

Abbreviations: AAVs: adeno-associated viruses; AhR: aryl hydrocarbon receptor; BMD: Becker muscular dystrophy; CAR: chimeric antigen receptor; CK: creatine kinase; DAMPs: damage-associated molecular patterns; DAPC: dystrophin-associated protein complex; DM: dermatomyositis; DMD: Duchenne muscular dystrophy; GPR35: G-protein-coupled receptor 35; IBM: inclusion-body myositis; IFNα: interferon-α; IL-2: interleukin-2; IMNM: immune-mediated necrotizing myopathy; KATs: kynurenine aminotransferases; NK: natural killer; NSAIDs: nonsteroidal anti-inflammatory drugs; PM: polymyositis; RICE: rest, ice, compression, and elevation; S1pr: sphingosine-1-phosphate receptor; scRNA-seq: single-cell RNA sequencing; TCA: tricarboxylic acid; Tconvs: conventional T cells; TCR: T-cell receptor; TNFα: tumor necrosis factor-α; Tregs: regulatory T cells; VAT: visceral adipose tissue

Introduction

The lineage-defining transcription factor of Foxp3$^+$CD4$^+$ regulatory T cells (or Tregs) was identified almost two decades ago [1–3]. This landmark co-discovery propelled Tregs from the shadowy realm of "suppressor cells" to the limelight of immunological research. It eventually became clear that Tregs control most types of immune reaction—including autoimmunity, allergy, inflammation, anti-tumor responses and anti-microbe responses—by regulating the activities of most innate and adaptive immunocyte types [4]. This superpower made Tregs and their products attractive candidates as immunotherapeutic agents [5, 6].

The therapeutic potential of Tregs was even further enhanced by the discovery of so-called "tissue-Tregs" [7]. During the initial decade after their discovery, essentially all studies on Foxp3$^+$CD4$^+$ T cells examined those circulating through the blood and lymphoid organs. The functional focus evolved from Treg impacts on other T cells, to effects on all adaptive immunocytes to influences on all immunocytes, whether adaptive or innate. In 2009, a unique population of Foxp3$^+$CD4$^+$ T cells was found in the visceral adipose tissue (VAT) of lean, "middle-aged" mice [8]. VAT Tregs have a transcriptome, T-cell-receptor (TCR) repertoire, and growth/survival factor dependencies that are distinct from those of their lymphoid-organ counterparts. Importantly, they control local and systemic inflammation and metabolism, at least in part by regulating the activities of local parenchymal [8] and stromal [9] cells. Subsequently, analogous populations of tissue-distinct Tregs were found at a multiplicity of sites, including skeletal muscle [10], skin [11], the colonic lamina propria [12], cardiac muscle [13], lungs [14], the liver [15], and the central nervous system [16]. These studies have revealed that the functional purview of tissue-Tregs extends well beyond protection from microbe and tumor challenges to tissue homeostasis at several junctures, e.g. regulation of metabolism, orchestration of tissue repair/regeneration, and regulation of stem/progenitor cell activities. Thus, the heightened promise of tissue-Tregs as immunotherapeutic agents rests on three points: (i) that recognition of local antigen(s) by their TCRs will encourage their accumulation at the site(s) where they are most needed; (ii) that specific accumulation at these sites will avoid the generalized immunosuppression

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or immunostimulation that systemic impoverishment or enrichment of Tregs can induce; and (iii) that their homeostatic activities will lend an additional therapeutic boost.

The options for performing Treg-based immunotherapy are manifold. This topic has been expertly reviewed quite recently [5, 6]; so we will just outline the major approaches here. As illustrated in Fig. 1, they can be grouped into four major classes. First, there are methods to preferentially elicit Foxp3$^+$CD4$^+$ cells *in vivo*: via injecting specific Treg growth factors [e.g. low-dose interleukin (IL)-2 or IL-2 variants], by administering inhibitors of molecules toxic to Tregs [e.g. dampening interferon (IFN)α or tumor necrosis factor (TNF)α], or through agonism or antagonism of receptors or co-receptors (e.g. agonism of TNFR2). The advantage of such strategies is that they avoid cell isolations and transfers, but their optimum application awaits the development of specific targeting methods in order to avoid systemic complications. Second are methods to expand isolated Tregs *ex vivo* and transfer them. The simplest strategy for expansion is CD3/28-bead-based expansion of polyclonal Tregs, but the low frequency of any particular Treg specificity in the blood renders this option relatively ineffective in most contexts. Antigen-specific Treg expansion ameliorates this problem although it introduces additional complexities–for example, which antigen? Third, methods for engineering more performant Tregs are becoming increasingly popular, sophisticated, and ingenious. These options may entail targeting a designated tissue by introducing a TCR that recognizes an antigen specifically expressed at that site or, alternatively, a chimeric antigen receptor (CAR) composed of an external antibody domain that binds to some molecule preferentially located there and an internal signaling domain that integrates into the Treg signaling network. And/or these options may involve synthetic augmentation of Treg performance, such as bestowing new chemokine or cytokine receptors or adding soluble mediator "payloads." Fourth are methods to *ex vivo* convert Foxp3$^-$CD4$^+$ conventional T cells (Tconvs) to Tregs. Transduction of FOXP3 is the most common variant of this strategy, often coupled with some sort of synthetic augmentation, as mentioned above. These so-called induced Tregs or iTregs have been the subject of some skepticism because they are thought to be less stable and less authentic than thymus-generated Tregs [17].

A few general concerns related to Treg-based therapies should be mentioned. One is the notion that Tregs might convert to pathogenic effector cells in the inflammatory lesion they are meant to control [18]. However, it is worth noting that evidence for this phenomenon, mostly derived from murine Treg-transfer models or from *in vitro* culture systems, has been down-played because the Treg preparations typically employed for such experiments are too-often contaminated with low levels of effector T cells or with cells not fully committed to the Treg lineage, a caveat that was, in general, not fully ruled out [19, 20]. Thus, employing highly purified, fully committed, thymus-generated Tregs or synthetic equivalents is prescribed for clinical applications. In addition, of the many ongoing and completed Treg-therapy trials, this issue has not proven problematic to our knowledge. A second point is the notion that Tregs depend on IL-2, which may be low or declining in the target lesion as therapy proceeds, and thus might have to be co-administered. This issue is likely to be most relevant to polyclonal Treg therapies and to survival of circulating Tregs as tissue-Tregs are known to be maintained, even expanded, by other growth factors, e.g. IL-33, IL-18 [7]. In addition, a recent clinical trial pointed out the dangers of co-administering IL-2 and Tregs, i.e. expansion of host T and NK effector cells [21].

As its title foretells, the focus of this review is the potential application of Treg-enhancing therapies to tissue regeneration. We have chosen skeletal muscle as a model system and will speculate on how the armamentarium of Treg-based treatments might be harnessed to ameliorate acute or chronic muscle pathologies. Current clinical trials in this area are limited and, thus, there is vast potential for this treatment strategy. Lastly, we will propose exercise as a natural Treg modulator.

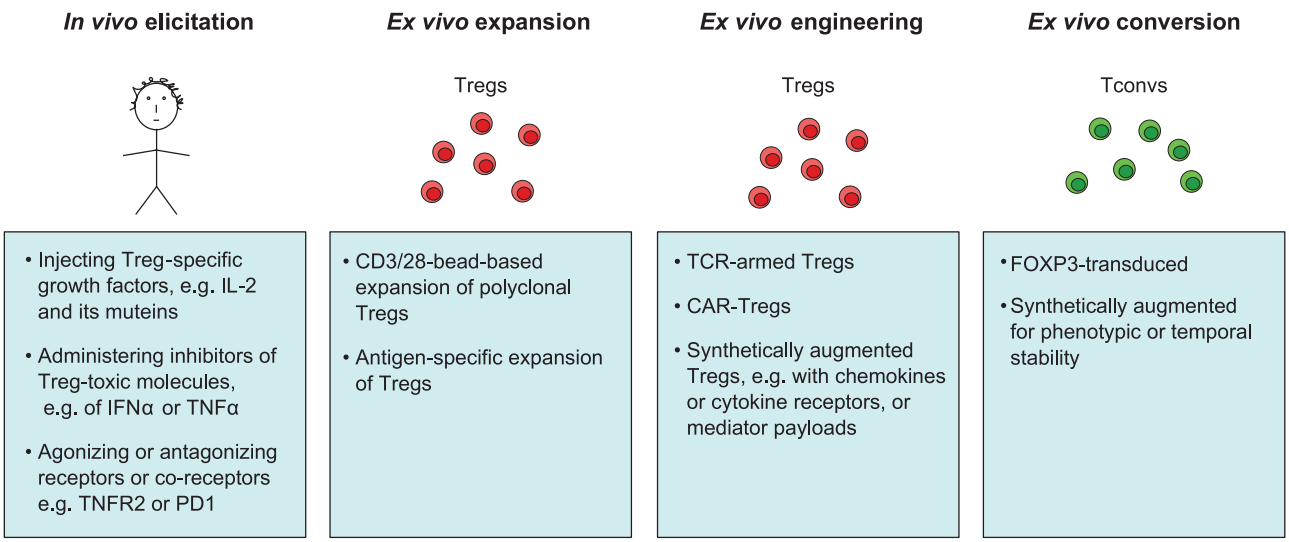

Figure 1: Treg-based therapeutic strategies. The increasing armamentarium of Treg-based treatments. See Introduction for details. Treg, Foxp3$^+$CD4$^+$ T cell; Tconv, Foxp3$^-$CD4$^+$ conventional T cell; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; R, receptor; TCR, T cell receptor; CAR, chimeric antigen receptor.

Treg therapies in acute muscle injury

Tissue regeneration is an orchestrated, multi-cellular process that follows a strict temporal order. Its dynamics are well characterized in the case of skeletal muscle. As the largest organ in the body, skeletal muscle is commonly injured due to a variety of reasons, including mechanical trauma, thermal stress, myo- or neuro-toxic agents, and ischemia [22]. Injury results in degradation and necrosis of myofibers. Replacement of damaged myofibers is initiated by a pool of muscle-progenitor cells (MPCs) located in close apposition to muscle fibers [23]. In response to acute injury, quiescent MPCs become activated, proliferate, differentiate, migrate and fuse to form new myofibers (Fig. 2) [24, 25]. In addition, early after injury, muscle mesenchymal stromal cells (MmSCs) get activated, proliferate, and form temporary extracellular matrix (ECM) that acts as a scaffold for the regenerating myofibers [26–29]. Remodeling and degrading ECM at later stages of regeneration is essential for efficient repair since excessive deposition results in fibrosis, impairing the restoration of normal tissue function and increasing susceptibility to re-injury [27, 30].

The inflammatory processes accompanying tissue injury also strongly influence the outcome of repair [31]. Early after injury, there is a rapid, transient influx of neutrophils (NFs) followed by pro-inflammatory macrophages (MFs), which are required for clearance of dead cells and cellular debris. In addition, injury invokes the rapid accumulation of various types of innate and adaptive lymphocytes, such as natural killer (NK), effector αβT, and γδT cells (Fig. 2) [32, 33]. The pro-inflammatory mediators produced by these accruing immunocytes—such as TNFα, IFNγ and IL-17A—are crucial for activating MPCs and trigging their proliferation [33–35]. Yet, the duration and magnitude of this initial inflammatory phase need to be tightly regulated since chronic exposure of MPCs to the same inflammatory mediators paradoxically blocks their differentiation into myocytes, resulting in impaired tissue repair [32, 36–38]. Additionally, uncontrolled inflammation results in fibrosis and scar formation [30]. Thus, within a few days of the insult, the muscle micro-environment transitions to an anti-inflammatory, pro-regenerative state. This shift is most pronounced for MFs, which switch to anti-inflammatory Ly6C^{low} phenotypes with various pro-regenerative functions, such as matrix remodeling and promotion of angiogenesis [39–42]. This dynamically and temporally regulated inflammatory response is a defining hallmark of efficient tissue repair that has been observed in a diversity of other tissues.

Over the last decade, Tregs became increasingly appreciated as tissular "rheostats" of muscle repair [10, 32, 43–45]. At steady-state, skeletal muscle harbors a small Treg population [10]. In response to acute injury, this population rapidly expands, reaching its numerical peak 3–4 days post-injury, a timepoint that marks a transition of the muscle milieu from a pro- to an anti-inflammatory state, before declining thereafter (Fig. 2) [10, 43, 44]. Treg accrual in skeletal muscle results from a combination of local proliferation, most pronounced 1 day after injury, and sphingosine-1-phosphate receptor (S1pr)-mediated emigration from lymphoid tissues [45]. Notably, decreased Treg recruitment to skeletal muscle after injury contributes to their diminished accumulation in aging animals [45]. The muscle microenvironment strongly influences muscle Tregs, as evidenced by their distinct transcriptional

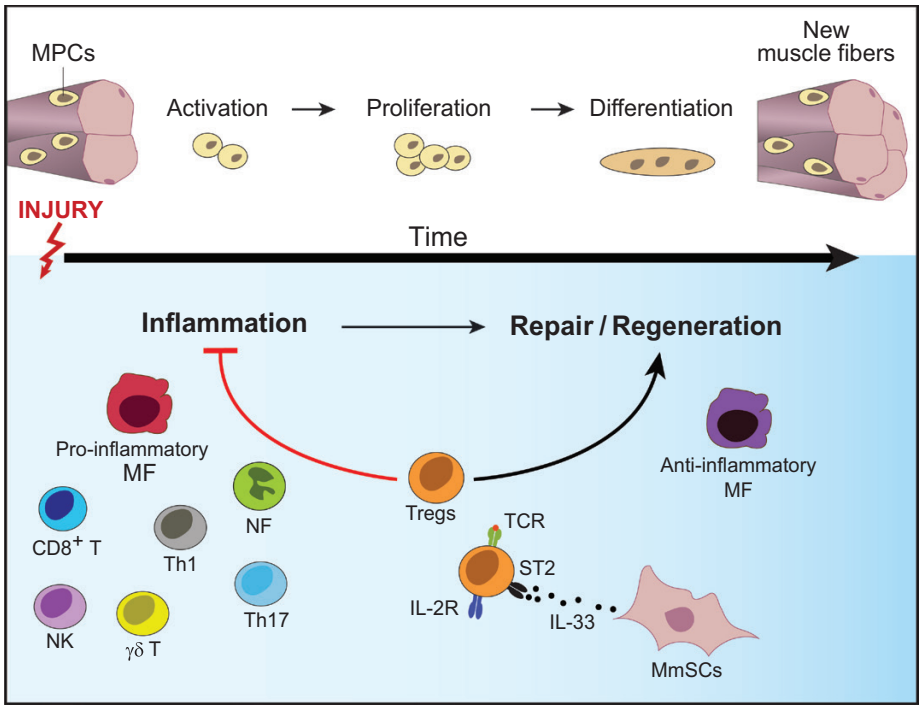

Figure 2: Tregs in skeletal muscle injury. In response to skeletal muscle injury, muscle-progenitor cells (MPCs) get activated, proliferate, and differentiate to form new muscle fibers (upper panel). Various immunocytes dynamically accumulate at the site of injury (lower panel), starting with neutrophils (NFs), followed by pro-inflammatory macrophages (MFs), natural killer (NK), γδT cells, CD8$^+$ T cells and T helper 1 (TH1) and TH17 cells. The subsequent reparative stage is dominated by anti-inflammatory MFs and Tregs that are essential for effective regeneration. Accrual of muscle Tregs in injured muscle is dependent on T-cell receptor (TCR) stimulation by local antigens, in addition to trophic cytokines, such as IL-33 produced by muscle mesenchymal stromal cells (MmSCs).

profiles compared with those of lymphoid-tissue Tregs [10, 46]. Muscle Tregs express a core set of nonlymphoid-tissue-Treg genes, including elevated levels of chemokine receptors, such as CCR2, CCR4, and CCR8 [10, 46]. The corresponding ligands for these receptors are rapidly upregulated by muscle stromal cells and infiltrating immunocytes after injury [47, 48], and therefore may aid Treg homing to the site of damage. Muscle Tregs also have an activated, effector-like phenotype, with preferential expression of key immunosuppressive molecules such as IL-10 and CTLA-4, which may arm them to control the strong inflammation induced upon injury [10, 43]. Compared with other tissue-Treg compartments, such as VAT or colon, muscle Tregs are distinguished by their continuous exchange with the circulating Treg pool [10, 46]. In addition, muscle Tregs have high proliferation rates at the early stages of injury, which is reflected in their upregulation of cell cycle and growth genes [10, 46].

The functional relevance of muscle Tregs in acutely injured muscle was demonstrated using genetic models allowing punctual Treg ablation, which exhibited compromised regeneration and tissue fibrosis, a consequence of inefficient repair [10]. Tregs employ at least two mechanisms to promote muscle regeneration. First, they control the inflammatory tenor of regenerating muscle by reining in immunocytes and promoting the pro- to anti-inflammatory shift in infiltrating myeloid cells [10]. In Treg-less mice, resolution of inflammation is impaired, with a persistent accumulation of NFs and inflammatory MFs. For example, Treg control of IFNγ production by NK and effector T cells is essential for controlling MF phenotype and function in regenerating muscle [32, 43]. Independently, Tregs exert their pro-regenerative power in a non-immunological fashion by directly interacting with MPCs and promoting their accrual. This effect is mediated at least in part by muscle Treg production of amphiregulin (Areg), a member of the epidermal growth factor family [10]. Thus, upon Treg ablation, MPCs exhibit reduced clonal efficiency, a deficiency that can be reversed by Areg administration [10]. Whether the anti-inflammatory and pro-reparative functions of Tregs are exerted by the same cells is currently under study. Single-cell RNA sequencing (scRNA-seq) analysis identified a considerable degree of transcriptional heterogeneity in muscle Tregs [46]. Intriguingly, muscle Treg subsets after injury are dynamic and evolve across the course of regeneration, raising the possibility that different Treg functions, such as dampening of inflammation and promotion of repair might be the roles of dynamically different Treg subtypes.

Treg accrual in muscle results from a combination of antigen- and cytokine-driven stimulation (Fig. 2). A characteristic of Tregs in acutely injured muscle is their restricted, clonally expanded TCR repertoire [10]. Intriguingly, one particular TCR clone was identified repeatedly in muscle Tregs isolated from multiple, independent animals, a strikingly rare incidence in the highly diverse TCR repertoires of different individuals [10]. This observation strongly suggests that muscle Tregs are responding to local antigens. Indeed, a transgenic mouse (tg) line harboring the rearranged transgenes encoding the TCRα and TCRβ chains of this particular clone (mTreg24 TCR-tg mice) shows enhanced Treg accumulation in injured muscle and improved muscle repair [49]. In addition to TCR-driven proliferation, the increase in muscle Tregs after acute injury, unlike that of their lymphoid-tissue counterparts, is strongly dependent on the alarmin cytokine, IL-33. Muscle insult induces rapid production of IL-33, primarily from MmSCs [45]. Treg-specific ablation of the IL-33 receptor (ST2) impairs their accumulation and hampers the regeneration process [45]. The IL-33/Treg axis is of high relevance in the context of muscle aging since diminished IL-33-dependent accumulation of muscle Tregs contributes, at least in part, to the regeneration deficiency in aging animals [45]. Disrupted IL-33-mediated Treg accrual in aging muscle is primarily due to decreased IL-33 production by MmSCs, and IL-33 supplementation boosts muscle Treg accumulation after injury and improves muscle repair [45].

Currently, treatment of acute muscle injury is limited to rest, ice, compression, and elevation (RICE), nonsteroidal anti-inflammatory drugs (NSAIDs), and physical therapy [22]. The objective of RICE is to minimize the size of the initial injury, inflammation, and subsequently the resulting scar. Yet, the impact of RICE has not been confirmed in randomized clinical trials [22]. While NSAIDs can offer analgesia and dampen inflammation, interfering with the early inflammatory process can hamper regeneration since NSAIDs can inhibit MPC proliferation [50, 51]. Chronic use of NSAIDs is also not recommended as it can cause serious gastrointestinal and renal side-effects, hypertension, and other systemic complications. The proposed ability of physical activity to promote efficient repair after acute injury is attributed, at least in part, to its immunomodulatory activity. We will elaborate on the role of exercise and immunocyte regulation via Tregs in an upcoming section.

Considering the limited therapeutic palette for acute muscle injuries, Treg-based therapies present a potentially more precise and effective alternative to traditional approaches. As highlighted in Fig. 1, enhancing Treg activity in regenerating muscle could potentially be achieved via the administration of Treg-trophic factors. Considering its preferential activity on tissue- (in particular, muscle) Tregs, IL-33-based therapies are likely to exhibit superior therapeutic specificity than IL-2 equivalents, which can cause systemic expansion of Tregs. IL-33 potently enhances muscle Treg accumulation [45], and Tregs expressing ST2 exhibit enhanced suppressive activity compared with that of their ST2$^-$ counterparts [52]. Yet, the effects of systemic IL-33 administration need to be thoroughly evaluated because of the multiple cellular targets of IL-33, including MFs, eosinophils, and type-2 lymphocytes [53]. An alternative approach, with better specificity towards tissue-Tregs, would be engineered fusion proteins of IL-2 and IL-33, which show therapeutic activity better than that of IL-2 and IL-33, alone or in combination, in models of nonlymphoid tissue inflammation [54, 55]. In light of the restricted TCR repertoire of muscle Tregs, administration of engineered antigen-specific Tregs targeting muscle antigens is a promising approach. Antigen-specific Tregs exert more potent suppressive activity in mouse models than polyclonal Tregs [56, 57]. Moreover, specific antigen-TCR interactions can promote Treg accumulation at the site of injury, as evidenced by the preferential accrual of Tregs from mTreg24 TCR-tg mice in injured skeletal muscle in comparison with other lymphoid and nonlymphoid tissues [49]. In addition to suppressing Tconvs targeting the same antigens, antigen-specific Tregs can exert bystander suppression of other T and non-T immunocytes at the site of injury [6]. The remarkable recent advances in gene-editing technologies offer the opportunity to arm muscle-specific Tregs with additional functional molecules with the potential to enhance tissue repairs, such as

IL-10 or Areg. In addition, modulating these Tregs to stably express particular transcription factors that foster their acquisition of the tissue-Treg program, such as BATF [58–60], can potentially enhance their homing and function in injured tissues. One pre-requisite for the success of Tregs engineered in this manner is identifying peptide antigens that can selectively activate and expand the muscle Treg compartment. So far, only a limited number of antigens recognized by Tregs at any location have been identified. Various approaches for scanning T-cell antigens have been successfully employed for the design of immunotherapies [61]. However, most of them have so far been employed to uncover ligands for $CD8^+$ T cells, in particular those recognizing limited viral proteomes. Recently, a peptide screen of VAT Treg antigens identified surrogate agonists that can specifically expand this population [62]. Employing similar approaches is likely to inform Treg cell therapies for acute muscle injuries.

Treg therapies in muscular dystrophies

Muscular dystrophies are a group of genetic diseases characterized by progressive skeletal-muscle weakness and degeneration. Within this group are Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD), both X-linked diseases caused by mutations in the gene encoding dystrophin (also known as dystrophinopathies) [63–65]. DMD is associated with the most severe clinical symptoms while BMD has a later onset and milder clinical presentation. The nature of these conditions depends on the amount of residual dystrophin in the muscle [66]. Disease severity is also related to the type of dystrophin mutation, with frameshift mutations associated with more severe disease (DMD) and mutations that preserve the reading frame with the less severe disorder (BMD), although some exceptions do exist [66–68].

Dystrophin is part of the dystrophin-associated protein complex (DAPC), a group of interacting muscle-fiber proteins that span the cytoskeleton, cell membrane, and extracellular matrix [69]. Dystrophin is located in the cytoplasm and links the intracellular actin network to the transmembrane element of the DAPC. A deficiency in dystrophin leads to the breakdown of the DAPC, which dramatically affects the structural integrity and contractile activity of skeletal muscle [70]. Over time, the muscle progressively degenerates and is replaced by fibrosis and fat, resulting in a devastating clinical course. DMD patients usually require a wheelchair by ages 10–12, need assisted ventilation around age 20, and eventually succumb to cardiac and/or respiratory failure between ages 20 and 40 [71].

In contrast to the acute toxin-induced injury discussed earlier, the pathogenesis of dystrophinopathies is multifactorial. Known contributors include the weakening of the sarcolemma, which is an important muscle-fiber structure that helps control mechanical stress during muscle contraction [70]. Another contributor is free-radical damage, as reactive oxygen, and nitrogen species are elevated in DMD [70]. Despite these numerous mechanisms, one major consequence and, subsequently, the contributor is the inflammation provoked by muscle damage [70, 72]. In the setting of acute skeletal-muscle injury, as was described in detail earlier, a response by the immune system is a typical and necessary correlate of muscle regeneration [73]. There is a highly dynamic and orchestrated reaction by numerous immunocyte types, which facilitates the regeneration process immediately after injury through normal tissue restoration [73]. However, in the context of DMD, there is a repetitive muscle injury, which results in chronic inflammation that exacerbates muscle damage [72]. The importance of inflammation in the pathogenesis of DMD has been highlighted by both human and mouse data. For DMD patients, systemic corticosteroids, which are strong immunosuppressants, have been the mainstay therapy for many years. Long-term corticosteroid treatment improves muscle strength and function, prolonging ambulation, and delaying pulmonary and cardiac dysfunction [74–76]. It also results in a reduction in the risk of secondary deficits such as scoliosis [74–76]. Muscle biopsies taken pre- and post-treatment in DMD patients given corticosteroids for 6 months showed a significant reduction in immunocyte numbers in the muscle tissue [77]. In mice, the *mdx* mutant strain, which harbors an alteration in the gene encoding dystrophin, is used as a genetic model of DMD. Depletion of specific myeloid and lymphoid populations substantially reduces muscle damage in this model [78–81]. Taken together, these mouse and human data underscore inflammation as an important driver in the pathogenesis of dystrophinopathies.

Tregs are a critical immunocyte subset due to their potent immunosuppressive activities. While typically a small population in skeletal muscle at homeostasis, Treg numbers are significantly elevated in *mdx* mouse and human DMD/BMD muscle [10, 43, 82]. Administration of an anti-CD25 mAb to *mdx* mice, used to deplete Tregs in this context because both dystrophin and Foxp3 are located on the X-chromosome, led to an increased inflammatory infiltrate in skeletal muscle according to both histology and an elevation in serum creatine kinase (CK) levels, an indicator of muscle damage [10, 43]. At the whole-tissue level, there was also an upregulation of transcripts encoding factors that promote fibrosis, a critical component of muscular dystrophy pathology [10]. A genetic mouse model permitting specific ablation of Tregs in *mdx* mice also revealed Tregs to be critical restraints on interferon IFNγ production by Tconvs [83]. IFNγ is pathogenic in *mdx* mice as it promotes a more inflammatory tenor in the muscle MF compartment and its genetic deletion resulted in reduced disease severity [83].

Given that inflammation has a major pathogenic role in muscular dystrophy and that Tregs exert numerous beneficial immunosuppressive influences in *mdx* mice, Tregs have the potential for the treatment of dystrophinopathies in humans. In comparison with corticosteroid administration, which results in global dampening of the immune system and severe adverse side-effects, Treg-based therapies can provide immunosuppressive activity with a predilection for sites of inflammation. As outlined in the Introduction, systemic low-dose IL-2 is an effective method for expanding Tregs *in vivo* [84]. In *mdx* mice, administration of IL-2/anti-IL-2 complexes (which extend the half-life of IL-2) leads to an increase in Tregs in the muscle but not in the spleen, which results in a dramatic decrease in skeletal muscle inflammation and lowers serum CK [10, 43]. The expression of IL-10, a key anti-inflammatory molecule, is increased in the muscle of so-treated *mdx* mice, although the functional importance of this increase was not established [43]. The fact that systemic Treg augmentation leads to selective enrichment of Tregs in the muscle and an associated increase in the level of muscle IL-10 argues for greater specificity of this therapeutic approach.

Interestingly, as was observed in acute muscle injury, $CD4^+$ T cells are clonally expanded in muscle tissue of both *mdx*

mice and DMD patients, indicating a response to one or more muscle antigens [10, 85, 86]. Indeed, when the *mdx* mutation was crossed into the mTreg24 mouse line, wherein T cells highly preferentially express α and β TCR chains from a Treg clone expanded in acute muscle upon injury, there was enhanced accumulation of Tregs in the skeletal muscle and improved muscle regeneration [49]. These data suggest a potential overlap between Treg clones and antigens found in chronic and acute models of injury in mice. With the identification of Treg clones expanded in human DMD muscle or of the antigens they are responding to, engineered Tregs with specific TCRs or CARs might prove to be an attractive therapeutic approach. Instead of simply boosting systemic numbers of Tregs, this approach would allow for the highly specific accumulation of Tregs within injured muscle and presumably more effective local immunosuppression and less systemic suppression. Although not yet studied in muscular dystrophy, Tregs from acutely injured muscle can also directly improve muscle regeneration through the production of Areg, which enhances myogenic differentiation, as noted earlier [10]. In the context of muscular dystrophy, one could postulate that transferred Tregs, in addition to suppressing the chronic inflammation, may have other undiscovered functions that directly enhance muscle regeneration, depending on the extent to which they can take on the mantle of true muscle Tregs after transfer.

Another aspect of employing Treg-based therapies for muscular dystrophy is their use in combination with approaches attempting to restore functional dystrophin in diseased muscle tissues. Currently, there are numerous dystrophin-restoring therapies in development: exon-skipping using antisense oligonucleotides to restore the reading frame in patients with out-of-frame dystrophin mutations; CRISPR/Cas9 editing to restore the reading frame of the dystrophin gene through directed DNA breaks; and adeno-associated viruses (AAVs) to deliver essential pieces of the dystrophin gene to the muscle [70]. While these are promising approaches, many delivery vectors can provoke immune responses [87]. Additionally, there have been numerous reports of dystrophin-specific autoreactive T cell responses in patients with DMD [88, 89]. These individuals do not produce full-length dystrophin protein and, as a result, do not sufficiently purge cognate self-reactive T cells during their thymic maturation and subsequent peripheral residence. Therefore, coupling dystrophin-restoring approaches with Treg-based therapies could allow for improvements in muscle function while limiting major side-effects associated with vector immunogenicity and dystrophin autoimmunity.

While DMD and BMD are major types of muscular dystrophy, there are numerous other diseases that similarly result in repetitive muscle damage and chronic inflammation. Limb-girdle muscular dystrophies are a diverse group of diseases caused by mutations in any one of more than 20 different genes, resulting in weakening and degeneration of the pelvic and shoulder girdle muscles [72]. While the exact nature of the inflammatory infiltrate has yet to be studied in-depth, patients have improved with corticosteroids [90]. Beyond muscular dystrophy, there are non-inherited inflammatory myopathies, a heterogenous group of diseases that share the common feature of immunocyte-mediated muscle injury. The most common diseases of this group are dermatomyositis (DM), polymyositis (PM), immune-mediated necrotizing myopathy (IMNM), and inclusion-body myositis (IBM). Our knowledge of the exact pathogeneses of these inflammatory myopathies is incomplete, but corticosteroids are often used and result in improved muscle strength.

Treg-based therapies aim to enrich Tregs at sites of inflammation, Tregs engineered with particular TCRs or CARs being the most specific, thereby resulting in localized effects. Although Tregs have numerous roles in skeletal muscle, given that an important function is immunosuppression, we speculate that Treg-based therapies could help control chronic inflammation with fewer side-effects and, thus, be applicable across a wide swath of muscle diseases.

Exercise as a natural Treg modulator

In the previous sections of this review, we highlighted Treg-enhancing therapies for the treatment of acute muscle injury and muscular dystrophies. Here, we will summarize what is known about the relationship between exercise and Treg activities and will propose mechanisms by which exercise may act as a natural Treg modulator. Finally, we will integrate these concepts with the prior sections to provide specific insight into how exercise may favorably impact the pathology of acute and chronic muscle injuries.

Exercise has been prescribed as an intervention to enhance health and stave off disease for millennia [91]. The concept of exercise as medicine is supported by a preponderance of evidence for an inverse relationship between physical activity level and all-cause mortality risk [92, 93]. It has been suggested that this relationship reflects the anti-inflammatory effects of exercise [94], which work to counteract the chronic inflammation associated with modern afflictions such as cardiovascular disease and type 2 diabetes. Indeed, such diseases arise from metabolic derangements in response to low physical activity and excessive nutrient availability, which is now known to impact the configuration and function of the immune system [95].

In addition to its direct effects on metabolic homeostasis via enhancing sensing and oxidation of nutrients by muscle and adipose tissues [96, 97], exercise has profound immunomodulatory potential. Schulz first documented this potential at the turn of the 20th century in a paper describing exercise-induced leukocytosis [98]. This phenomenon has been attributed to increased blood flow and elevated concentrations of catecholamines and cortisol [99]. Indeed, β-adrenergic blockade achieved via propranolol administration attenuates exercise-induced leukocytosis [100]. In contrast, lymphocytopenia occurs in the period of recovery after exercise cessation and persists for 24–48 h [99, 101]. Although early reports attributed this effect to increased apoptosis, it is more likely to reflect increased lymphocyte extravasation into peripheral tissues. Recent studies measuring apoptosis and the expression of adhesion molecules such as CD18, CD53, and CD54 on circulating immunocytes after exercise support this interpretation [102–104]. Furthermore, high-intensity exercise, especially modalities involving loading during the lengthening (eccentric) phase of muscular contraction, results in myofibrillar disruptions [105, 106] and myocellular release of damage-associated molecular patterns (DAMPs) such as mitochondrial DNA, ATP, Tenascin C, and HMGB-1 [107]. These factors may activate muscle-resident stroma and immunocytes, leading to the formation of chemokine gradients that would attract cells mobilized to the blood during exercise [108]. Notably, the magnitude and

duration of lymphocytosis and lymphocytopenia in response to exercise are dependent on exercise intensity. The age, sex and training history of the organism as well as the mode, duration, and frequency of exercise are additional variables affecting the reported immunomodulatory effects. For a summary of innate and adaptive blood immunocyte responses to exercise, the reader is referred to two excellent reviews on exercise immunology [109, 110].

Changes in Treg frequency, number, and function in peripheral blood in response to various human and rodent exercise regimens have also been the subject of recent reviews [111, 112]. Although these reviews highlighted many acute and chronic exercise interventions that augment the representation of Tregs in the circulation, there are also many studies documenting no change or diminished Treg counts. These discrepancies are almost certainly consequences of heterogeneity in the cohorts tested, exercise regimens used, and the times at which blood samples were collected in relation to exercise. Given the immediate increase and subsequent reduction in Treg representation in peripheral blood after exercise [101], the time of analysis is of critical importance. Yet many studies have taken only one pre- and one post-exercise sample, and some chose a very early post-exercise timepoint (<24 h), while others looked only late into recovery (>3 d). Given the role of local Tregs in responses to acute and chronic muscle injury discussed in the previous sections of this review, it is possible that studies documenting reduced Treg presence in the blood late during exercise recovery are merely looking where Tregs sojourned on their way to sites of need. Taking this perspective, one would expect lower Treg counts in the blood after prolonged, high-intensity exercise capable of inflicting significant tissue damage, such as a marathon race, and this result is what has been documented [101, 113]. Further support for this perspective comes from analyses demonstrating increased proportion and function of Tregs in non-muscle peripheral tissues of exercised vis-à-vis sedentary mice after experimental injury [114–116]. Aside from histological observations of inflammation in intensely exercised rat and human muscles [105, 117, 118], there is a dearth of information on immunocyte activities in skeletal muscles after exercise. Fluorocytometric analysis of paired blood and muscle samples after exercise of various durations and intensities will be critical for finding a definitive answer to whether exercise modulates muscle-Treg numbers and functions. Despite the paucity of studies looking at skeletal-muscle immunocytes after exercise, there are a few well-documented exercise-induced adaptations that may potentially support Treg accumulation and function in muscle. We propose that increased local lactate concentration, increased production of the tryptophan metabolite kynurenic acid (Kyna), and changes in the gut microbiota are three mechanisms by which exercise might modulate muscle Treg numbers and/or functions.

During intense exercise, carbohydrate metabolism is the dominant metabolic pathway used to fuel ATP production to sustain muscular work [119]. As exercise intensity increases, the production of lactate exceeds the rate of pyruvate oxidation in the tricarboxylic acid (TCA) cycle, which leads to an accumulation of lactate in myofibers and in the muscle extracellular space. This high-lactate environment, although painful for the athlete, may suit Tregs well: studies comparing the metabolic phenotypes of Tconvs and Tregs have shown that the latter is more dependent on oxidative phosphorylation [120, 121] and can use lactate in low-glucose environments as a source of pyruvate to fuel TCA cycle and electron transport chain activities to support suppressive function [122, 123].

Exercise also enhances the production of the tryptophan metabolite Kyna via PGC-1α-dependent upregulation of kynurenine aminotransferases (KATs) [124, 125]. Kyna is a ligand for the aryl hydrocarbon receptor (AhR) and G-protein-coupled receptor 35 (GPR35) [126]. Interestingly, muscle injury increases the expression of GPR35 and AhR on Tregs several fold [10], and AhR signaling promotes the generation of Tregs in other contexts [127, 128]. Kyna-mediated modulation has been proposed for circulating Tregs [111, 112]; it will be interesting to explore this mechanism in muscle-localized Treg populations.

Finally, exercise training produces significant changes in the gut microbiota [129–132]. Remarkably, 6 weeks of endurance exercise increases *Faecalibacterium* and *Lachnospira* communities while decreasing *Bacteroides* in the guts of lean human participants [129], and chronic endurance exercise promotes an elevation in short-chain fatty acid production in the gut [129, 132]. Changes in the microbiota influence Treg generation and phenotype in the gut via microbe-dependent metabolites [133–135], and Tregs traffic between the gut and extra-gut tissues [136]. Therefore, changes in the gut microbiota could be a potential mechanism of Treg modulation by long-term exercise.

As alluded to above, muscle injuries are commonly treated according to the RICE principle, which was first introduced in 1978 [137]. However, the American College of Sports Medicine now suggests a gentle movement of an afflicted area within the first 24 h after injury, followed by progressively challenging physical activity after 48–72h. Furthermore, controlled muscular contractions elicited in a previously injured area by transcutaneous electrical nerve stimulation is a common practice in physical therapy for treating acute and chronic muscular injuries. Thus, although there are no randomized clinical trials comparing post-injury immobilization to exercise, the widespread implementation of movement-based therapies after muscle injury by practitioners of sports medicine and physical therapy raises the question of whether exercise might be both the "poison" and the cure for sports-related muscular injuries. We propose that, through the mechanisms described above, exercise might mobilize Tregs to then home to injured sites to support the transition from pro-inflammatory to pro-repair processes. Furthermore, exercise might be prophylactic against excessive inflammatory responses subsequent to acute injury by increasing the number of Tregs in muscle.

Exercise as a therapy for muscular dystrophies was once avoided due to the "work overload" theory, which predicted deleterious effects on muscle function. However, *mdx* mice allowed to exercise voluntarily for several weeks do not display worsened hindlimb muscle, diaphragm, or cardiac muscle pathology [138, 139]. Instead, exercise-trained *mdx* mice, have improved hindlimb muscle function compared with sedentary *mdx* mice, despite running significantly less per day than age-matched healthy mice [140, 141]. Cardiac function in dystrophic mice is also improved by voluntary exercise [139]. Impressively, a meta-analysis of studies investigating the effect of exercise on muscular dystrophy patients found a significant association between exercise and improvement in endurance during walking [142]. It will be useful to know whether these benefits of exercise coincide with Treg

modulation. Indeed, if exercise augments Treg representation and function in healthy and *mdx* muscles, then the mechanisms mediating exercise-induced Treg modulation may be elucidated and used in active or sedentary individuals to facilitate muscle repair. Thus, exercise or exercise-based treatments tailored to modulate Treg activities may be a novel therapeutic approach to treating acute and chronic muscle injuries.

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None.

Conflict of Interest

DM is a cofounder of TRexBio and is a cofounder, member of the Scientific Advisory Board, and member of the Abata Advisory Board of Abata Therapeutics.

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Data Availability

Not applicable. This paper did not generate new data.

Author Contributions

All authors contributed to the literature review for and to the writing and revision of this article.

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Not applicable.

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