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Short Review

Normal and Aberrant Muscle Tissue Healing, Learning from Health and Disease

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Special Issue: Molecular Mechanisms of Skeletal Muscle Wasting and Weakness in Different Pathophysiological Conditions

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Abstract

Human skeletal muscle exhibits remarkable plasticity, being responsive to chemical, mechanical, metabolic, and inflammatory stress. When the homeostatic disturbance is below a threshold of significant damage, the muscle responds by modifying metabolic activity, cell size/shape, and structure, thereby normalizing cellular function. If the disturbance causes significant damage, skeletal muscle, along with a precisely choreographed response from the immune system, can regenerate. Very few pathological conditions inhibit these adaptive responses in muscle. Yet, from these few conditions, we can learn a great deal. Working with the immune system, normal muscle healing can inform disease treatments, and the disease pathology informs our understanding of normal muscle healing. Here we use Duchenne Muscular Dystrophy (DMD) as a model of failed muscle adaptation/regeneration to attempt to understand normal muscle healing, why it sometimes fails, and how normal muscle response might be applied to understand and treat DMD.

Keywords
Muscle; dystrophy; regeneration

1. Introduction: Muscle Healing and Dystrophinopathy

Human skeletal muscle is a remarkable tissue in that it is highly responsive to mechanical and chemical stimuli and has a unique and high regenerative capacity after injury, relative to most other human tissues. During regeneration after injury, the repaired muscle correctly aligns its functional cells and connective tissue in response to stimuli (mechanical and chemical) that occur during the repair and remodeling process. Skeletal muscle cells can also undergo substantial changes in cell size and metabolic capacity in response to stimuli that disturb its homeostasis, e.g. “exercise training”, but are below the threshold to induce significant structural damage [1, 2]. This ability to repair and respond to a plethora of insults (plasticity) have led the tissue to be dubbed colloquially by some as “plastic fantastic” [3]. This adaptive response of skeletal muscle relies on a complex interaction with the immune system. The interaction occurs when responding even to a low stress stimulus and especially in response to significant damage. The repair and response process is tightly coordinated amongst several immune cells and relies upon diverse cellular actions that occur in a precise sequence [4]. The system is fairly robust in that very few human pathologies inhibit the exercise or muscle repair response [5-7]. Yet, a small handful of genetic mutations that occur in the human population expose some weak points in the system. Mutations in structural proteins can be particularly problematic for skeletal muscle, which is not surprising considering the constant physical stresses placed upon it during normal daily human activities. Herein we will describe and compare one particular genetic muscle disease relative to the normal muscle repair process to better understand the disease itself in muscle and potential treatments, and also better understand the normal muscle repair process.

Duchenne muscular dystrophy (DMD) is an X-linked recessive skeletal muscle disorder, affecting approximately 1 in 3600 males during early childhood. DMD causes the boys to be wheelchair dependent most often around the age of 10-12 with a life expectancy of between 20 and 40 years due to severe progressive cardiomyopathy and/or progressive degeneration and fibrosis of respiratory muscles [8]. DMD is caused by a mutation in the dystrophin gene that leads to a complete absence of dystrophin protein. The protein is an intracellular protein with functions in cell signaling, coordination of multi-protein structures, and mechanically stabilizes the sarcolemma of muscle fibers. Dystrophin provides a link between the intracellular cytoskeleton and sarcolemma through the dystrophin-associated protein complex (DAPC) [9]. DAPC, a large transmembrane protein complex, can absorb shock during muscle contraction, and receive and transduce cellular signals [10]. A lack of dystrophin leads to an inability of the DAPC complex to assemble, thus losing some signaling and structural functions. The sarcolemma becomes highly susceptible to physical damage leading to continuous contraction-induced membrane damage followed by inflammation, repair, and regeneration. The regenerative capacity of myofibers is eventually diminished and becomes exhausted, leading to myofiber necrosis, chronic inflammation, fibrosis, and fat deposition [10-13].

The treatments for DMD can be generally divided into two categories: (1) dystrophin-targeted therapy, such as modified gene delivery, genome editing, and protein replacement therapies, and (2) pathology-targeted therapy, such as long-term corticosteroid use and histone deacetylase inhibitors. Although these treatments showed beneficial effect in DMD, the side effects and nonspecific action of them are inevitable [14-16]. In order to understand DMD disease progression and pathology better and find more effective treatments for DMD patients, studies utilize various animal models including nematode worms (C-elegans), zebrafish, mice, and dogs. By far the most common animal species is the mouse for which several strains exist with the most common being the mdx. This model presents a DMD skeletal muscle phenotype due to a spontaneous point mutation causing no functional dystrophin protein, similar to the human condition. However, the life span in mdx is reduced by ~25%, while in human DMD it is reduced by ~75% [17]. The skeletal muscle in mdx mice is indistinguishable from the wild type mice in the first 2 weeks after birth; but the onset of necrosis is abrupt between 3 to 6 weeks after birth; and then begins a relatively stable phase [17]. The onset of disease progression in mdx mice is similar to DMD in humans, but the ultimate fate of muscle fibers can be quite different. This suggests that dystrophin deficiency is a conditional factor instead of a determinant factor [18]. Understanding the other determining factors will greatly enhance our understanding of the disease and will inform future therapeutics. Due to the amount of research conducted on the mdx model, comparisons can be made between the plethora of old and the new research in this model, making the comparison a valuable part of current dystrophic research. The complex interactions between dystrophin deficiency and other factors including inflammation, physiologic milieu, or fibrosis is a key to DMD progression. Indeed, 50% to 80% of the muscle damage of the disease has been purported to be caused by inappropriate activation of the immune system [4, 19]. To better understand the aberrant muscle repair process in DMD, we will clarify and summarize the normal muscle repair process first.

Healthy muscle repair itself is a complex process that at least partly recapitulates the embryonic growth of muscle through the sequential expression of myogenic transcription factors. Adult muscle stem cells, also called satellite cells, are a necessary cell group of muscle regeneration, they express transcription factors that drive activation, proliferation, and differentiation of these cells to form new muscle fibers. Although skeletal muscle fiber hypertrophy is not dependent on satellite cells, such as overload-induced muscle hypertrophy, satellite cells are essential in muscle regeneration and assist most normal muscle repair [20]. Muscle repair with satellite cells involvement can be broadly divided into three phases based on the time-course following muscle injury, destruction and clearance phase, activation of myocytes phase, and remolding phase. The first phase (1-7 days) includes necrosis of damaged myofibers and removal of damaged cellular debris, which is mainly caused by initial damage and then driven further by the inflammatory process. The activation of myocytes phase (7-14 days) includes activation, proliferation, and differentiation of the muscle stem cells. At the end of the remodeling phase, the new myofibers form, muscle regeneration is achieved, tissue alignment finishes, and muscle repair stops [21, 22]. Recent studies revealed that there is a complex and surprising level of coordination between the muscle and immune system [23-25]. Therefore, we will emphasize the process of tissue healing in light of the activation of the immune system due to the relatively consistent pattern between muscle inflammation and regeneration [4, 19], (Figure 1).

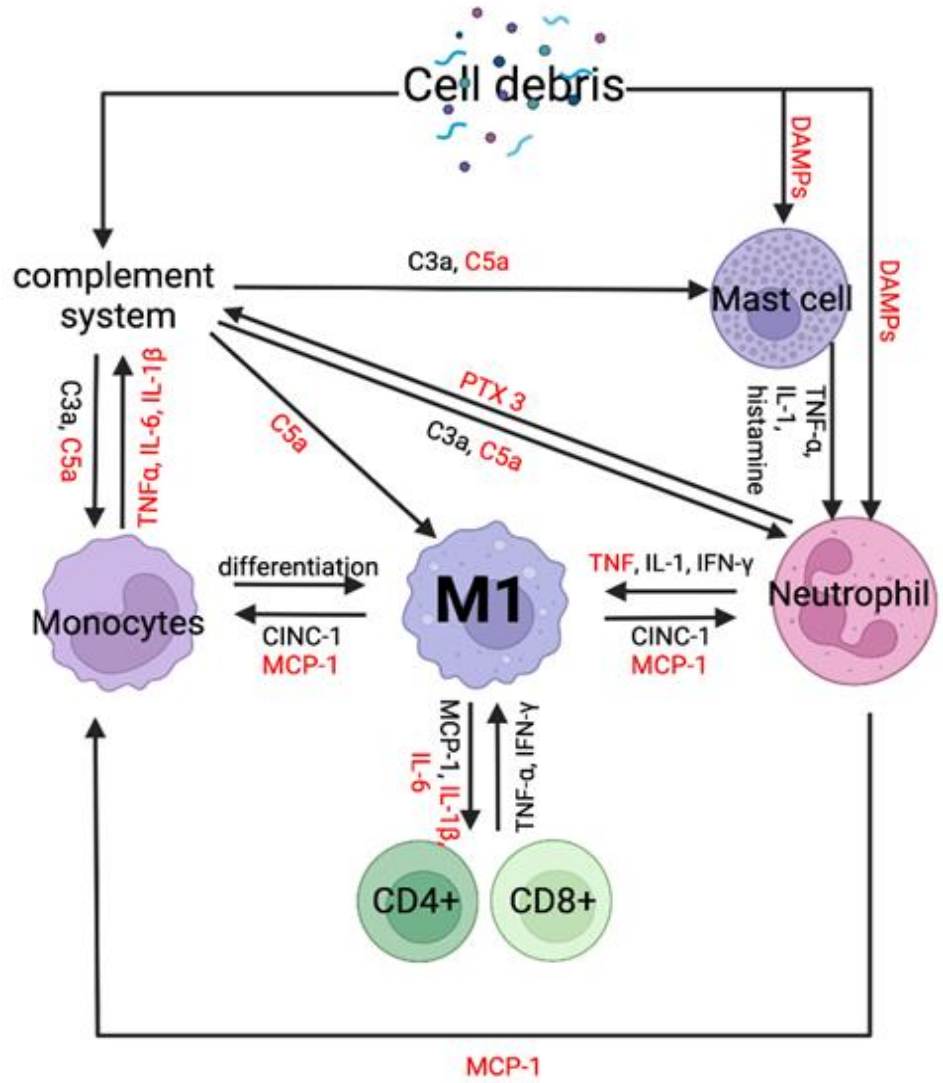

Figure 1 Muscle cell/tissue healing process coordinated by the immune system**.** Several immune cells coordinate the muscle repair process. Those factors in RED are dysregulated in Duchenne Muscular Dystrophy and thus help to inform areas of potential treatment. DAMP (Damage Associated Molecular Pattern), IL- "#" (Interleukin-number), C3a (Complement-3a), TNF (Tumor Necrosis Factor), PTX (Pentraxin), CINC (Cytokine-induced Neutrophil Chemoattractant), MCP (Monocyte Chemoattractant Protein), M1 (Macrophage type-1), IFN (Interferon).

The muscle repair process in DMD is initially similar to normal muscle repair and includes inflammation and regeneration. However, the muscle repair process in DMD never resolves. Within an entire skeletal muscle such as the vastus lateralis for example, as one section of the muscle is in the regeneration stage, another nearby collection of muscle cells is beginning muscle necrosis, and another may be in remodeling. This has been referred to as asynchronous muscle healing [26]. Due to the continuous damage and the immune cells in various phases of the inflammation/healing process communicating with each other, it is thought that signals become confused. For example, a macrophage may be receiving cytokine signals that suggest tissue necrosis and early stages of inflammation and also muscle remodeling signals at the same time. This can result in macrophages expressing M1 and M2 phenotypes simultaneously and seems to contribute or exacerbate the

constant inflammatory state. This not only inhibits effective repair but appears to exacerbate the muscle damage pathology. In the first phase, the inflammatory process in DMD is over activated and prolonged because of the mutation in the dystrophin gene and a weaker cell membrane. This constant damage process would make satellite cells activate continually and then possibly lose their proper function. At the end of the remodeling phase, the muscle regeneration is not complete and muscle repair cannot conclude. The ongoing discoordinated inflammation becomes a major contributor to the pathology.

2. The First Phase of Injury: Destruction and Clearance (1-7 Days)

Normal muscle injury manifests as a decrease in muscle force production or disruption of muscle fiber structure, which is normally caused by lacerations, contusions, normal muscle use, or overload. Following muscle injury, the cell debris and cell content leakage from the damaged muscle site initiates the inflammatory response in the absence of any microorganisms. This sterile inflammatory process drives muscle regeneration by recruiting leukocytes such as mast cells, neutrophils, macrophages, and lymphocytes to the site of injury from the local tissue or from the blood. Here we discuss the characteristics and functions of various cells involved in the inflammatory process based on the timeline beginning with the main non-cellular component of the immune system, the complement, and then examining individual cell types (Figure 2). Where an important distinction or similarity between animal and human studies exist, we highlight these points.

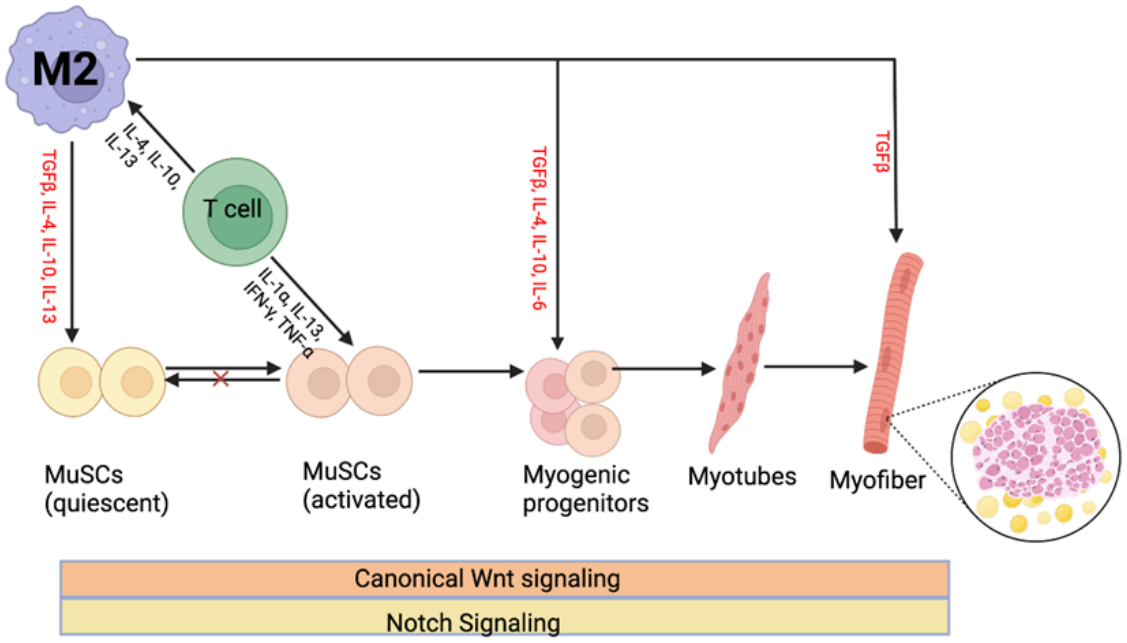

Figure 2 Late stage muscle repair process coordinated by the immune system**.** This figure highlights the normal muscle repair process that is driven primarily by M2 macrophages. Dysregulated factors are highlighted in RED and appear to make the process pathological in Duchenne Muscular Dystrophy. MuSC (Muscle Stem Cells), TGF-B (Transforming Growth Factor Beta), IL- "#" (Interleukin-number), TNF (Tumor Necrosis Factor), IFN (Interferon).

2.1 Complement

The complement system, as a part of innate immune defense, consists of soluble proteins in the serum, which can be activated and lead to a cascade of proteases. Through various recognition molecules, the complement system is activated via three different pathways: the classical pathway, the lectin pathway, and the alternative pathway [27, 28]. Recent experiments found the complement system can be activated by muscle cellular debris and lead to a cascade of proteases via the classical and alternative pathways during muscle injury, but the lectin pathway is still unclear. In a modified reloading muscle study, Frenette et al. used soluble form of complement receptor-1 (sCR1) to inhibit complement activation at different time points post injury, they found fewer neutrophils and macrophages are recruited in the experimental group (complement activation inhibited) than in the control (normal complement activation) at 6 hours, but similar at 24 hours [29]. This demonstrated that complement system was affecting the repair process at these time points. C3a and C5a, are the two major products of complement cascade, they promote the adhesion and/or migration of neutrophils and macrophages to the lesion by P-selectin, E-selectin, intercellular adhesion molecule-1, and integrins [14, 30-32]. C5a can also increase the complement receptors (C5aR) on monocytes and macrophages, in turn increasing the expression of TNFα, IL-6, IL-1β [33, 34]. Further experiments suggest that histamine from mast cells also interacts with C3a and C5a through the receptors (C3aR and C5aR) on mast cells [35, 36]. The mechanism, however, behind the complement-mediated activation of mast cells is still unclear in skeletal muscle tissue. In one of the most recent discoveries of complement in muscle repair, the complement component 4b was identified as a modulator of muscle loss with aging and a regulator of muscle repair [37]. The authors speculate that this could introduce new therapeutic avenues as new methods to modify complement emerge. Regardless of therapeutics, the activation of the complement system does appear to be an important step in muscle repair by recruiting and activating various immune cells during the inflammatory and repair process.

The complement system repair process appears to be dysregulated in dystrophic muscle. A recent study used genome profiling to identify DMD biomarkers in humans. In DMD patient muscle biopsies compared to healthy control, C3 was identified as a top candidate biomarker [38], suggesting that this early phase of muscle repair had not resolved. In animal dystrophic muscle, Hyzewicz et al. found an elevated level of the complement cascade product C5a in mdx [39]. Through utilizing C5a blocker, they found inhibition of C5a can reduce necrosis and circulating monocytes. However, the expression of myeloperoxidase (MPO) and number of macrophages is increased in muscle by C5a blockade. This result could be because that inhibition of C5a decreases circulating monocytes but increases movement of monocytes into the tissue, which is supported by Cote et al. [40]. They found the enhanced proliferation of macrophages after monocyte depletion is due to triggering resident precursor cells within skeletal muscles. Further studies will clarify the specific mechanisms between C5a, macrophages, and monocytes in dystrophic muscle. In this regard, a recent study found that mdx mice had higher PTX3 than control mice in skeletal muscle [41]. PTX3 is an inflammatory mediator affecting complement activation, is produced by neutrophils, endothelial cells, and fibroblasts and can act as double-edged sword decreasing neutrophil recruitment in localized inflammation but enhancing leukocyte recruitment and activating complement system in systemic inflammation [42]. However, there is no current study examining the connection between PTX3, complement system, and neutrophils of skeletal muscle in DMD.

Both human and animal studies support the role of dysregulated complement in dystrophic pathology, yet few details or molecular pathways have been elucidated. An improved understanding of these pathways in muscle is likely to lead to at the least, an adjuvant anti-inflammatory treatment in DMD. This seems especially relevant considering that a top biomarker is from this very early phase of muscle repair. Targeting the dysregulation at an early stage would likely produce a cascade of corrective events in the pathology. In the next step, after complement activation, some of the first leukocytes recruited/activated are mast cells during the pro-inflammatory phase.

2.2 Mast Cells

Mast cells (MC) often found in low numbers of tissue resident immune cells play an important role in muscle repair. Mast cells are a type of granulocyte derived in bone marrow, circulates in the blood, but do not fully mature until recruited or activated in the tissue at the site of injury. After injury, mast cells are activated primarily through damage-associated molecular patterns (DAMPs) around the injury site, which stimulates degranulation. Degranulation is an index of activation of mast cell, which extrude the cytoplasmic granule contents into the extracellular space by exocytosis. A significant increase of neutrophil density was found by utilizing a mast cell-degranulating agent (CMP 48/80) from 6-24 hours after injury, suggesting that mast cells can increase neutrophils recruitment at the beginning of inflammation [43]. Mast cells have a large number of cytosolic vesicles, which release pro-inflammatory mediators such as histamine and tryptase in muscle repair. Tryptase, a serine protease is only found in mast cells and released after activation of the cell. Duchesne et al. analyzed mast cell density and tryptase at time points after muscle injury. They found two peaks of tryptase expression: 6 h-12 h after injury and at 96 h after injury, but density of mast cells not change until 72 h after injury [44]. Thus the effect of mast cells during pro-inflammation may be due to the enhanced function of mast cells instead of the increased density. Tryptase is also shown to stimulate myoblast proliferation via protease-activated receptor-2 (PAR-2) in vitro, this is supported by the second peak of tryptase expression (72 h after injury) in the Duchesne at al. experiment. But this mechanism has not been confirmed in vivo. Future studies will need to isolate the function of tryptase on myoblast proliferation in vivo (typically occurring 3-4 days after injury) and extend the timeline to at least 7 days after injury.

In DMD, mast cells have not been studied extensively, but can be activated by mechanical myofiber damage. Compared to the number of mast cells in normal muscle injury, mdx mice and DMD patients have 3-fold more mast cells and produce more histamine and proteases in the injured muscle [45]. This was further supported by the experiment of Radley et al. as well [46]. Radley et al. utilized cromolyn, a mast cell stabilizer, to inhibit mast cell degranulation and analyze the effects of cromolyn treatment in mast cells in mdx mice. They divided them into three groups: cromolyn injected group, PBS injected group, and untreated mdx group. They found the density of mast cells in the untreated mdx group during 21 to 28 days after birth had no change, but the degranulation of mast cells decreased at 21 days and 23 days in the cromolyn injected group. These results suggest that compared to the normal muscle injury group, the number of mast cells in mdx group is continuously at a higher level, but only has higher degranulation at certain time points. This continuous high level of mast cells could possibly increase neutrophils recruitment, and cause extended inflammation. Ideally, future studies can add another wild-type group for direct comparison to healthy repair, and modify mast cells in mdx in specific time periods, and pay

attention to decreasing the density of mast cells instead of their function as mast cells appear to be playing some role in dystrophic pathology.

2.3 Neutrophils

Neutrophils, the first of the white blood cells to extravasate after tissue injury, play a role in healthy and abnormal tissue repair. In the early stage of muscle regeneration, the main function of neutrophils is to clear necrotic cellular debris. Neutrophils are the predominant immune cell derived from myeloid stem cells in bone marrow, making up 40% to 70% of all white blood cells. They are the first non-resident cells recruited to the site of injury and play multiple roles in muscle regeneration. In addition to the effect from the complement system, damaged and necrotic cells are also responsible for early neutrophil recruitment. These damaged cells are responsible for the damage-associated molecular patterns (DAMPs) including DNA, histones, high mobility group protein B_1 ($HMGB_1$), Adenosine triphosphate (ATP), interleukin-1α (IL-1α) and other molecules [47]. Neutrophils express G-protein-coupled receptors (GPCRs) that recognize DAMPs, which assist activation and chemotaxis [48]. Additionally, DAMPs can activate the production of lipid mediators that are derived from arachidonic acid [49]. These lipid mediators are also strong inducers of neutrophil chemotaxis and can be recognized by GPCRs in the neutrophils. From 6-24 hours after injury, neutrophils are the most abundant immune cell in damaged muscle tissue, secreting TNFα, IFN-γ (interferon-γ), and IL-1β (interleukin-1β) [50]. These cytokines secreted by the local neutrophils continue to induce infiltration of peripheral neutrophils from blood. Additionally, the neutrophils produce superoxide which increases muscle membrane lysis in the injury area [19]. Therefore, the neutrophils enhance the clearing of cellular debris and phagocytosis by secreting cytokines and attracting other leukocytes during the first two days after injury.

In DMD, neutrophils are also recruited through DAMPs and cytokines. The injured muscle in DMD generates more DAMPs, cytokines, and immune cells in the damaged site because of the extensive and continuous damage [13, 51]. These excessive DAMPs are recognized by toll-like receptors (TLR) that promote the neutrophil release of myeloperoxidase (MPO) and neutrophil elastase (NE). The MPO not only increases oxidative stress to promote muscle cell lysis but also activates the nuclear factor kappa B (NF-κB) pathway. NF-κB is considered one of the major pathways leading to chronic inflammation in DMD and induces pro-inflammatory gene expression such as cytokines, chemokines, and enzymolysis [10, 13]. Henriques et al. supported this mechanism through utilizing the TLR blocker. They found blockade of TLRs in mdx was effective at reducing symptoms of inflammation [52]. Another driving factor in increasing inflammation in DMD is elevated secretion of TNFα. Hodgetts et al. analyzed the necrosis area in the untreated mdx group, neutrophil-deletion mdx group, and blocked TNFα in mdx mice [53]. They found the necrosis area significantly decreases when utilizing pharmacological blockage of TNFα or depletion of neutrophils at 21 days after birth. This result suggested that in mdx mice neutrophils and TNFα are contributing to the pathology, and decreasing TNFα or neutrophils is another effective way to reduce the cytotoxic inflammation. Additionally, they found in the untreated mdx group, the onset of skeletal muscle necrosis was abrupt at 21 days, then greatly reduced at 24 days, and remains low level after 24 days. This further clarifies the main time period of neutrophil function being early after birth in mdx mice. Focusing on this time period in future studies in mdx could further delineate the specific role of neutrophils in mdx muscle pathology. Additionally, neutrophils can also secrete monocyte chemoattractant

protein-1 (MCP-1), which can recruit monocytes to the injury site [54]. A human study examining muscle biopsies of children with DMD found that muscles express increased levels of MCP-1 associated with a higher expression of IL-17 [11]. This elevated MCP-1 is likely related to the increased neutrophils in dystrophic muscle. More studies that examine the role of neutrophils at different stages of the disease would contribute to our understanding of these cells in the pathology of DMD and the relationship between neutrophils, MCP-1, and monocytes.

2.4 Monocytes (Macrophages)

Monocytes appear to be the longest-lived white blood cell in muscle tissue during normal muscle healing and of DMD patients. They typically circulate in the blood for 1 to 3 days and then migrate into damaged tissues where they differentiate into macrophages. A subset of circulating monocytes will crawl against the blood flow, extravasating rapidly to the injury area, secreting tumor necrosis factor-α (TNF-α), and upregulating other genes of the cytokine system and further activating complement [55]. Just a few hours after injury, these patrolling monocytes are the main producer of TNF-α which will promote the recruitment of other leukocytes. Macrophages are critical regulators in skeletal muscle regeneration [56], coming from monocytes, after crossing the endothelium of capillaries into damaged tissue via the CCR-CCL axis and complement C3a-C3aR axis [50]. Macrophages are often classified in classically activated pro-inflammatory macrophages (M1) (activated by LPS) and alternatively activated anti-inflammatory macrophages (M2) (activated by IL-4). After acute injury, activated macrophages secrete cytokine-induced neutrophil chemoattractant 1 (CINC-1) and MCP-1 and increase recruitment of neutrophils and monocytes. Then the TNF-α, IL-1, IFN-γ produced by complement system and neutrophils increases infiltration of macrophages. At 2 days after injury, M1 macrophages peak in muscle lesions, phagocytose cell debris, and express TNF-α and IL-1β [21, 57, 58]. The TNFα and IL-1β will further increase the neutrophils function. From 4 to 7 days after injury, M1 macrophage activity gradually declines and these cells shift phenotype to M2 macrophages. M2 macrophages generally secrete IL-10 which is anti-inflammatory but also TGF-B, which in excessive amounts leads to extensive fibrosis. The M2 phenotype also inhibits satellite cell proliferation further contributing to decreased muscle repair.

Currently macrophages are probably the most intensively studied immune cell in DMD at least partly because they are the most abundant inflammatory cell in dystrophic muscle [59, 60] and because modifying muscle macrophage infiltration attenuates disease pathology [59, 61]. Several extensive reviews are available on this topic and so we only give an overview of the topic here [62-64]. The muscle injury in DMD is amplified 2 to 5-fold by leukocytes, especially by macrophages [19]. M1 macrophages are continuously recruited and produce high inflammatory cytokines such as TNF-α, IL-1β, and IL-6. The inflammatory cytokines drive production of inducible nitric oxide synthase (iNOS) which amplifies muscle membrane damage, cell lysis, and delays muscle regeneration [13, 59, 65]. Additionally, excessive TNF-α and IL-6 can upregulate this NF-κB pathway and promote inflammation further in DMD and inhibit satellite cell differentiation to myotubes. The macrophage dysregulation appears to contribute to the asynchronous tissue damage and repair, which is known to exacerbate the pathology, and possibly contributes to the issue of trained immunity that we discuss later. Macrophage activity continues to be an area in normal muscle tissue repair on and DMD pathology. As macrophages transition, T-cells become more active in the pro-inflammatory process.

2.5 T Cells

T cells are immune cells derived from lymphoid stem cells in bone marrow and mature in thymus gland. Matured T cells can be divided into two main types: Cytotoxic T cells (CD8+) and Helper T cells (CD4+). They are present and active during an extended time in the muscle regeneration process. Fu et al. utilized cardiotoxin (CTX) to induce a muscle injury in the Rag-/- mice model which lacks both T and B cells [66]. They found the group with transplant-activated CD4+ and CD8+ had a lower number of myofibers with centralized nuclei and larger myofiber size compared to the untreated group at 7 days after injury. This demonstrates CD4+ and CD8+ are significant for MuSCs (Muscle Stem Cells) and facilitate muscle regeneration. This result is also supported by Zhang et al.'s study [67]. Zhang et al. investigated the role of CD8+, and the interactions between CD8+ and macrophages via a CTX-induced skeletal muscle injury model [67]. They found the peak of CD8+ at 3 days after injury and were nearly absent at 14 days, the CD8+ mainly function during the 3-14 days after injury. This study also found CD8+ KO group has lower concentration of MCP-1 and fewer M1 macrophages, which demonstrated CD8+ can regulate M1 macrophages via MCP-1. Additionally, because CD8+ deficient (CD8+ KO) group had smaller newly formed myofibers than the wild-type (WT) group, it suggests that CD8+ plays a role in muscle regeneration. Castiglioni et al. also investigated the function of T cells in a CTX-induced skeletal muscle injury model [68]. They utilized flow cytometry to identify which population of T cells is involved in muscle regeneration. However, they did not observe infiltrating cells expressing the CD8 marker from 1 to 7 days after injury.

Foxp3+CD4+ regulatory T cells (Tregs) are derived from CD4+ T. They are a group of cells that can suppress inflammatory process and modulate muscle repair and regeneration and have three main functions. Firstly, Tregs can decrease the infiltration and activity of CD4+ and CD8+. Secondly, Tregs can inhibit infiltration and promote apoptosis of neutrophils by secreting IL-10 during the beginning of muscle injury (3 days after injury) [69, 70]. Additionally, Tregs can stimulate M1 macrophages to polarize to M2 macrophages via IL-4, IL-10, and IL-13. Recent studies found that the CD4+ T cells had higher frequency in injured muscle also because of the accumulation of Tregs and distinct T cell receptors (TCR) bind with muscle Tregs [71, 72]. Depending upon the extent of injury, after infiltration of immune cells have begun clearing necrotic tissue, some of these same cells participate in the next phase of healing which is activation of resident cells, especially satellite cells, to complete tissue repair.

Several animal studies suggest that T cells play a role in dystrophy pathology [73, 74] and that the role may primarily be in driving fibrosis with less effects on other areas of muscle pathology [75]. A more recent study has however demonstrated a more extensive role of T cells in dystrophin pathology. Comparing the histological structure of the thymus gland (essential for T cell maturation), significant structural abnormalities and cellular locations were noted in the mdx mouse. When the thymus glands from mdx or control mice were transplanted into nu/nu mice, the several muscle pathology features were evident in mice receiving the thymus from mdx mice [76]. Several human studies also suggest a role of T cells in dystrophic pathology. Corticosteroid treatment has been shown to reduce the total number of T cells in biopsies of patients with DMD and also reduce the number of muscle fibers invaded by T cells [77]. In a study of 75 DMD patients, blood T cell levels were shown to correlate with disease severity and progression [78]. Peripheral blood levels and biopsies of patients have further reported substantial variability which at the very least appears related to the disease, although notably the variability in biopsy T cell infiltration is more than that

of macrophages [79]. A final consideration of T cell activity in DMD relates to potential autoreactivity of a patient's T cells to a newly introduced version of dystrophin protein whether it is from dystrophin delivery or exon-skipping induced truncated dystrophin this will result in a novel protein expression in the patient for which their T cells have not been trained to ignore. At least three studies have examined possible autoreactivity of a patient's immune system with epitopes of dystrophin. Estimates range from 8% to 53% of patients will express some form of autoreactivity to dystrophin, which appears to be influenced by glucocorticoid treatment [80-82]. This could result in mild to severe side effects and may require testing of patients before treatment with a dystrophin replacing intervention. Although less appreciated in dystrophic pathology compared to macrophages, understanding T cell activity will be critical in developing future treatments.

3. The Second Phase of Muscle Tissue Healing (7-14 Days)

3.1 Activation and Proliferation of Muscle Stem Cells

Satellite cells are also referred to as muscle stem cells (MuSCs) to differentiate them from the specific glial cells also named satellite cells in the brain. MuSCs are precursors to skeletal muscle cells which are necessary for the growth, maintenance, and regeneration of skeletal muscle [83]. MuSCs have unique characteristics due to their gene expression and location. They express a canonical biomarker paired box transcription factor Pax7, located beneath the basal lamina of myofibers. In mature resting muscles, MuSCs are mitotically quiescent due to the predominance of the Notch signaling through expressing Sprouty1 (Spry1) and miR-708 [84]. After muscle injury, the Notch signaling weakens but Canonical Wnt signaling enhances. MuSCs can be activated due to downregulation of Sprouty1 and upregulate myogenic regulatory factors MRF (e.g. Myf5 MyoD) [83, 85]. Several studies found there is an intermediate status between the quiescent and activated state, called the "alerted" state, (G_{alert}), which can be activated by damaged tissue through mTORC1 (mammalian target of rapamycin) signaling [86, 87]. G_{alert} MuSCs have higher mitochondrial activity and regenerative capacity compared to the quiescent MuSCs. The activation and proliferation of MuSCs peak from 4 to 7 days after injury (Figure 3). During this time, M1 macrophages gradually shift to M2 macrophages, which is stimulated by insulin growth factor (IGF-1). M2 macrophages activate MuSCs by secreting transforming growth factor-β (TGFβ), IL-4, IL-10, and IL-13 [88]. The interaction between these cytokines and the transition from Notch to Wnt signaling plays a major role in the normal activation and proliferation of MuSCs. Additionally, several other proteins have proven to affect the signaling as well. For example, ADAMTS1 (A Disintegrin-Like And Metalloproteinase With Thrombospondin Type 1 Motif) secreted by macrophages can increase MuSCs activation by inhibiting Notch signaling [89]; a group of extracellular proteases called MMPs (matrix metalloproteinases) can regulate skeletal muscle regeneration via Notch and Wnt signaling in mdx mice as well [90].

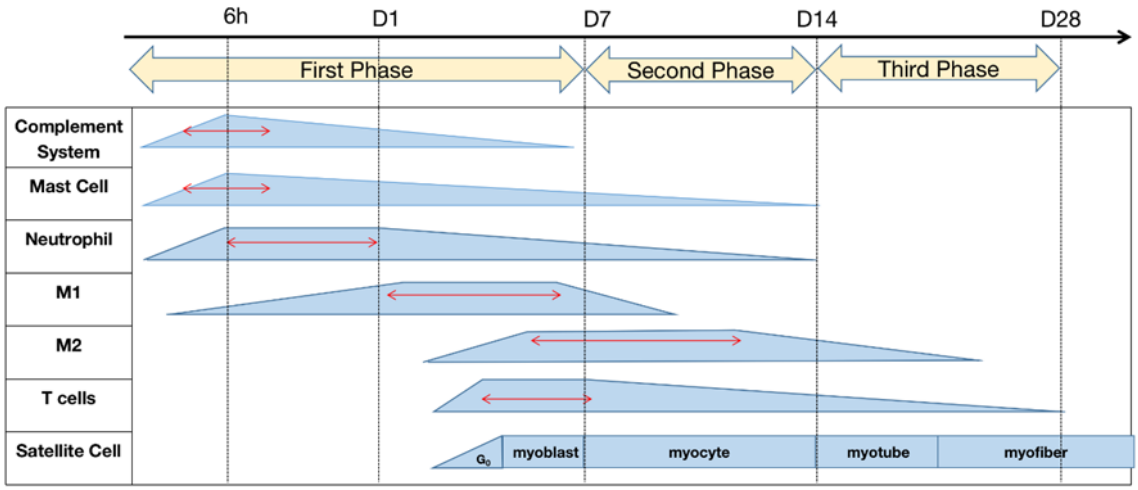

Figure 3 Normal muscle healing timeline. The normal muscle healing timeline highlights the sequential and coordinated nature of the response of the immune system in muscle repair. Red arrows indicate time of peak activity for each cell or process. M1 (Macrophage type 1), M2 (Macrophage type 2), G (Cell cycle phase G0 or quiescence)

T cells also affect normal MuSCs activation and proliferation. Fu et al. examined the effect on MuSCs of 13 types of cytokines that were shown to be secreted by T cells [66]. They found (IL-1α, IL-13, IFN-γ, TNF-α) can promote MuSC proliferation and expansion, and maintain their undifferentiated status both in vitro and vivo. Castiglioni et al. found sustained expression of FOXP3 from 1 day to 15 days after injury, which means Tregs might affect the activity of MuSCs [68]. They then utilized myogenic differentiation assays to test the expression of Pax7 in the presence of in-vitro derived Treg cells (iTreg), natural Treg group, naive CD4+, and activated non-polarized CD4+. They found the expression of Pax7 increases but myotube formation decrease in the iTreg group. This result suggested Tregs can increase the number of MuSCs and stimulate MuSCs proliferation, but inhibit MuSCs differentiation and myoblast fusion. Future studies might isolate different cytokines in the presence of Tregs to understand the mechanism of the effect of Tregs on MuSCs and attempt to more closely examine these effects in human cells. Although it is still unclear the mechanism behind effects of mast cells on MuSCs proliferation, some studies demonstrated that mast cells can stimulate myoblast proliferation relying on tryptase at 4 days after injury [44, 91, 92].

3.2 Differentiation, Fusion, and Maturation of the Muscle Stem Cells

Activated MuSCs can repair and replace damaged cells by going through asymmetric division and symmetric division. After asymmetric division, one satellite cell can generate a committed myogenic progenitor cells (differentiation) and a new satellite cell retains stem cell characteristics [85]. These myogenic progenitor cells also (myoblasts), can give rise to myotube through fusion and ultimately into myofibers through maturation. The differentiation, fusion, and maturation of satellite cells are essential in normal muscle regeneration. During the differentiation of satellite cells, M2 macrophages secrete TGF-β, IL-10, IL-6, and IL-4 which increases myoblast fusion and promote myotube formation [93-95]. Asymmetric satellite cell divisions are also controlled by various factors, such as Notch signaling, Par complex, myogenin and dystrophin [96-100]. In normal muscle healing, myogenin and Par complex are the prerequisite for myogenic differentiation due to their association

with MyoD [101]. MRFs (e.g. Myf5 MyoD) promote differentiation of myoblasts through repressing Pax7 and Pax3 transcription. Finally, dystrophin is a regulator to segregate Mark2 and Pard3 to opposite ends of the cell, which can promote asymmetric division through retaining satellite cell polarity [97].

3.3 Muscle Stem Cells in DMD

In the absence of dystrophin, unstabilized sarcolemma can lead to chronic muscle injury and repeated rounds of damage, inflammation, and regeneration. This constant damage process caused by chronic inflammation recruits more M2 macrophages producing TGFβ, and requires more myogenic progenitors [13, 102], (Figure 4). However, constant regeneration can exhaust satellite function for proliferation and repair. Kottlors et al. found DMD muscle has more Pax 7 expression than healthy control through muscle biopsy in DMD patients, suggesting that DMD muscle has a higher number of quiescent or activated satellite cells at some stages [103]. The result is also supported by several mdx studies [97, 104, 105]. Additionally, one study confirmed that dystrophin deficiency in satellite cells of mdx mice can reduce the polarity of MuSCs, which then resulted in a reduction of asymmetric division [97]. This demonstrates the impaired regeneration in dystrophic muscle is not simply due to the exhaustion of the SC pool but also the dysfunction of MuSCs. A recent genome-scale metabolic analysis in human DMD myoblasts appears to confirm these findings [106]. Because of the constant damage process caused by chronic inflammation, more M2 macrophages are recruited. M2 macrophages then aberrantly secrete elevated levels of IL-4, IL-10, and TGFβ. Madaro et al. examined the effect of macrophages in MuSCs by using Diphtheria Toxin (DT) or PBS in specific mdx mice (ITGAM-DTR mice crossed with mdx mice, whose macrophages can be depleted by DT injection) [107]. They found the DT group had less PAX7 expression, which demonstrated that macrophage depletion could inhibit MuSCs proliferation, leading to exhaustion of the SC pool in mdx. However, they found the DT group had higher Myog expression, indicating it had more myoblasts and an early onset of differentiation. Additionally, IL-10 and TGFβ have been confirmed in rescuing differentiation defects of MuSCs in ex-vivo animal studies [19, 107]. Taken together, macrophage is a key factor to change the proliferation/differentiation potential of MuSCs in DMD. Future studies can examine the mechanism of these cytokines in mediating the interaction between MuSCs and macrophages in vivo, and provide new insights into therapeutic approaches in dystrophic muscles. And while replication in human DMD samples is ideal from a scientific perspective, there are numerous ethical and practical reasons why they are difficult to obtain, and verification might be more practically sought through replication in animal models to inform our understanding of human dystrophy.

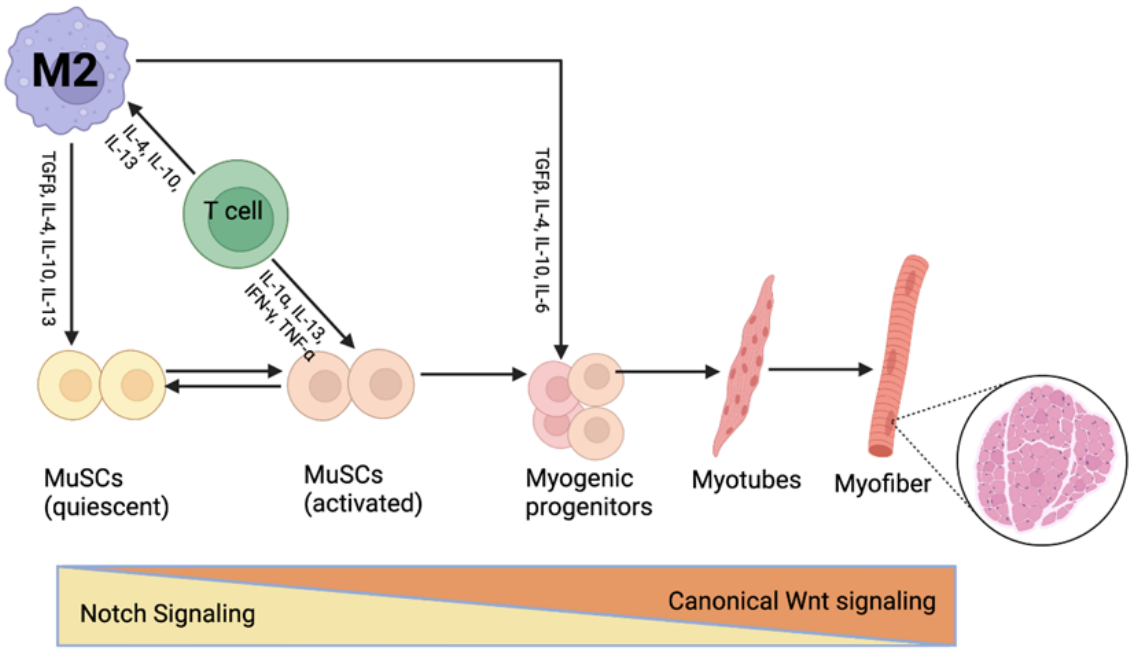

Figure 4 Coordination of immune cells to drive regeneration of muscle tissue. M2 macrophages drive the timing of signaling pathways during skeletal muscle regeneration through coordination with T-cells to drive the proliferation and differentiation of satellite cells. M2 (Macrophage type 2), MuSC (Muscle Stem Cells), TGF-B (Transforming Growth Factor Beta), IL- "#" (Interleukin-number), TNF (Tumor Necrosis Factor), IFN (Interferon)

Besides the immune response, the complex dystrophic environment can also dysregulate cell polarity, and impair asymmetric cell division, reducing myogenic potential [108]. The absence of dystrophin can decrease the expression of Mark2, inhibit asymmetric division, and delay muscle regeneration [97]. Additionally, matrix metalloproteinases (MMPs) are increased in dystrophic muscle in several studies [90, 109, 110]. Hindi et al. found the higher expression of PAX7 and increased Notch signaling, when inhibiting MMP-9 in mdx mice [90]. These results showed the effects of MMP-9 on MuSCs in dystrophic muscle, and suggested inhibition of MMP-9 can improve MuSCs proliferation. Additionally, they also found the inhibition of MMP-9 can elevate IL-4 but reduce IFN-γ and IL-6, which also confirms their anti-inflammatory effects.

A complicating factor in understanding satellite cell activation in dystrophy is the mdx model itself, which as mentioned has reasonably high regenerative potential. Yet the human satellite cell activity eventually decreases. A recent report by Mazala specifically address this issue [111]. The mdx mouse, while being the most common model, has a milder phenotype than humans. A newer mouse model utilizes the same genetic mutation of dystrophin on the DBA/2J (D2) genetic background. These mice have a more severe phenotype that more closely mirrors the human pathology and disease progression. In this study, they compared the early regenerative capacity, through activated satellite cells, of mdx to D2 mice. The D2 mice had a substantially reduced capacity to activate satellite cells, for example 30% of satellite cells of mdx mice were Brud U positive 24 hours after injury, as compared to 6% of satellite cells in D2 mice. This appeared to be at least partially caused by TGF-B and local Fibro-adipogenic progenitor (FAP) cells. Blocking signaling of TGF-B improved pathology in D2 mice and slightly increased the activity of satellite cells. It is likely

that an interaction between FAP, muscle, and immune cells is coordinating the inhibition of regenerative capacity. Immune cell involvement was not a direct outcome in this study but future studies should examine this interaction. Even as therapies improve, satellite cell exhaustion will likely still be a problem in at least some patients so understanding the process and how interventions like anti-inflammatories or exercise, both known to modify inflammation, interact with gene therapies will be important.

4. The Third Phase of Muscle Tissue Healing: Remodeling; and Long-Term Maintenance and Therapeutics

4.1 Remodeling

As inflammation is decreasing and new myofibers are being formed or completely repaired, muscle fibers will align along the axis of physical stress being placed upon the muscle. New connective tissue formation, begun during myoblast proliferation, is now also being aligned with the muscle fibers. In some repair instances of excessive or continuous inflammation, the production of connective tissue is excessive and can even begin to replace functional muscle cells; this process is known as fibrosis. Fibrosis can thus be defined as excessive extracellular matrix (ECM and connective tissue) and is a unique hallmark of DMD. Although connective tissue is essential for muscle repair, their excessive accumulation can cause severe pathophysiology effects. In dystrophic muscle, elevated TGFβ secreted by M2 macrophages can increase connective tissue production by fibroblasts. Additionally, elevated TGFβ can decrease the production of ECM-degrading enzymes but increase inhibitors of ECM-degrading enzymes such as inhibitors of metalloproteinases (TIMPs) [102]. However, the effect of elevated TGFβ only during the early stages of the disease, while fibrosis continues to progress later [19, 112]. Therefore, there is another mechanism related to fibrosis dependent on the sustained secretion of M2 macrophages. The M2a macrophage phenotype appears almost at the same time as M1 macrophages in DMD. In normal muscle healing, M1 macrophages secrete nitric oxide (NO) by utilizing arginine to induce inducible nitric oxide synthase (iNOS). However, arginine can be also metabolized by arginase in M2 macrophages, producing ornithine. Ornithine can be further metabolized to L-proline, and ultimately becomes connective tissue [19, 113]. Wehling et al. found elevated expression of arginase in M2 macrophages in mdx, which suggests a role of M2a macrophages in dystrophic muscle pathology [59]. Some studies hypothesized the increased availability of arginine for arginase is due to the reduction of nNOS in dystrophic muscle. More studies are needed to understand the mechanisms among arginine, nNOS, iNOS, and arginase before treatments can be directed to modify this pathway.

4.2 Emerging Issues in Muscle Repair, Dystrophy, and Rehabilitation

Rapid progress is being made in the understanding of skeletal muscle repair in healthy muscle in response to injury. However, as highlighted here, the interaction of the muscle repair process with the immune system is complex. For diseases like muscular dystrophy that include a pathologic component of chronic inflammation, this interaction is especially complex and some recently emerging issues appear to further complicate this process. Namely these are 1. the precise sequential activity of immune cells, 2. trained immunity, 3. As gene therapy improves, the increase in physical activity and physical therapy, and 4. adjuvant/complementary therapies. Immune cells

and cytokines enter into and function in muscle repair in specific sequential nature and the underlying stage of pathology appears to disrupt the sequence. For example, IFN-gamma is known to aid muscle repair in healthy muscle or early in the dystrophy disease process, yet at later stages, appears to impair recovery [114]. Further, cytokines such as leukemia inhibitory factor appear to worsen inflammation in early disease but then decrease inflammation and fibrosis in late stage disease [115, 116]. Thus manipulating inflammation is likely to be disease stage dependent and would affect nearly all therapies [62]. A second issue is trained immunity. Repeated activation of immune cells by DAMPs, as occurs continuously in DMD, can train immune cells to react in an exaggerated way in response to tissue injury and this appears to occur in dystrophic mice [62, 117]. Thus, the innate immune system is no longer acting with precise sequence to drive optimal tissue repair. Third is the variety of gene therapy interventions and how each will react with increased physical activity. As these therapies improve, patients (especially young boys) are very likely to increase physical activity and exercise. This is likely to be a very complicated topic because it will depend on the treatment itself, such as full-length dystrophin, micro-dystrophin, or exon skipping. Even the micro-dystrophin which has proven very successful in mice can use different gene promoters (with each for example reacting differently to exercise stimulus) and have different levels of distribution throughout the muscles of the body. Furthermore, at what stage of disease is the treatment first delivered and then specific dosing of exercise in terms of form of exercise, intensity, regularity, and recovery will interact with the diverse gene therapies [118]. Recent reports suggest exercise will not be detrimental and will likely assist muscle recovery and function [119, 120]. But again, the variability of treatments could affect the outcome. Finally, because few treatments purport to return dystrophin levels to healthy control muscle in 100% of muscle fibers, some form of disease will still exist. Thus, anti-inflammatories and other adjuvant therapies are still likely to be of benefit but it is too early in the research process to discern which might be the most effective. As we continue to examine the new treatments about the normal and aberrant muscle repair process of diseases like muscular dystrophy, the learning will occur in both directions. The interactions of gene therapies with the immune system in this disease continue to teach us about the disease and normal muscle healing, while ongoing experiments in normal muscle healing continue to inform the new disease treatment experiments. We are currently in a phase of rapid advances in understanding muscle tissue healing. This holds much promise for effective therapies but our understanding of the complexity of the tissue repair process ensures that many questions remain unanswered.

5. Conclusions

DMD is caused by an absence of a structural protein which leads to muscle cell damage and the initiation of inflammation. In a healthy muscle, this damage might be similar to everyday use, and inflammation would coordinate to drive tissue repair. Yet in DMD, the muscle inflammation/muscle repair process becomes dis-coordinated and instead exacerbates the pathology. Simply dampening inflammation produces benefit but does not appear to allow the immune system to restore or resynchronize the repair process. Suppressing the immune system thus dampens destruction but does not restore repair. Most promising therapies either restore dystrophin expression through viral delivery of a micro-dystrophin protein or attempt to restore dystrophin expression through a truncated protein (exon-skipping). These approaches cause the production of a protein that is not native to the patients' body and the immune system has not been trained to disregard it, potentially

complicating the interaction with the immune system. It seems unlikely that any one, standalone therapy will be able to fully treat the disease. Thus, continued improvement of our understanding of the healthy muscle repair process (especially when combining exercise) will be necessary to completely understand how therapies could and should interact. With several clinical trials ongoing, and continuous research in healthy muscle repair and tissue regeneration, we stand to make rapid progress in the coming decade in treatments for DMD.

Author Contributions

M. Kostek was responsible for concept development, writing, and editing. S. Liu was responsible for figure creation, writing, and editing.

Competing Interests

The authors have declared that no competing interests exist.

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