SKELETAL MUSCLE IMMUNOMETABOLISM

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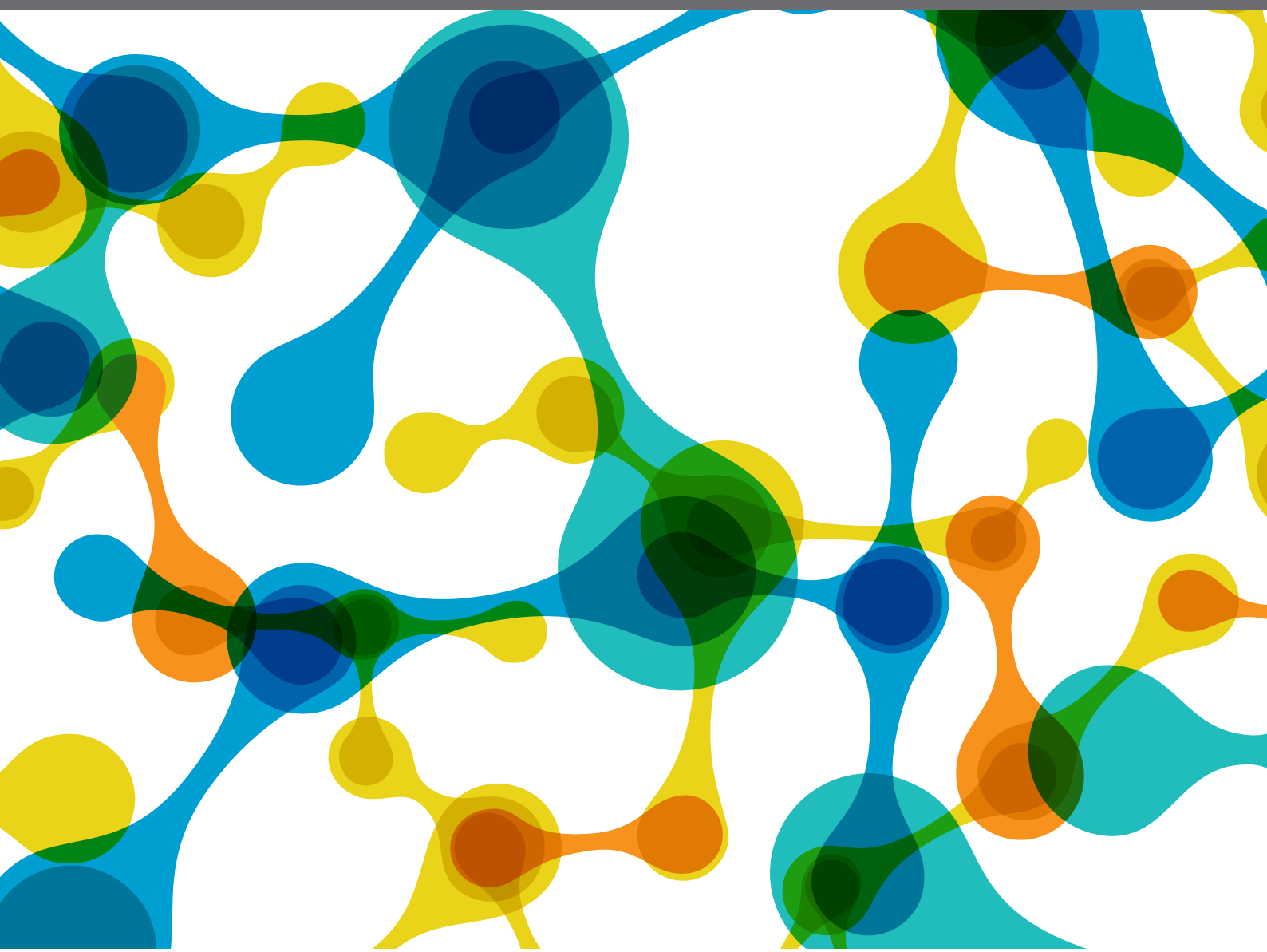

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SKELETAL MUSCLE IMMUNOMETABOLISM

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EDITORIAL
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Editorial: Skeletal Muscle Immunometabolism

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Editorial on the Research Topic

Skeletal Muscle Immunometabolism

INTRODUCTION

Skeletal muscle inflammation is triggered by multiple physiological and pathological processes. Resident immune cells such as macrophages and dendritic cells respond to the inflammatory state of skeletal muscle, and circulating immune cells can be recruited to muscle tissue (Pillon et al., 2013). Skeletal muscle adaptation depends on sequential stages of degeneration, inflammation, and regeneration (Chazaud, 2016). This remodeling process results from a finely tuned orchestration of cellular, molecular and metabolic responses involving both muscle and non-muscle cells (inflammatory cells, endothelial cells, fibro-adipogenic cells, pericytes). If unbalanced, this response leads to muscle atrophy, and/or fibrosis. This Research Topic on immunometabolism incorporates reviews and original studies to elucidate the many implications of skeletal muscle inflammation in metabolism, health, and disease.

SKELETAL MUSCLE-IMMUNE CROSSTALKS

The inflammatory response needed for optimal muscle adaptations involves crosstalk between the immune and non-immune cells. Bonomo et al. summarize the interactions between macrophages, dendritic, and T-cells in inflammatory responses associated with idiopathic inflammatory myopathies, Duchenne muscular dystrophy, and muscle regeneration. The review by Howard et al. describes the orchestration of the inflammatory response mediated by satellite cells and immune cells during skeletal muscle recovery from injury. The activation state of immune cells attracted to skeletal muscle is paramount to adequately trigger pro- or anti-inflammatory responses, and the use of glycolysis or fatty oxidation as the primary energy source influences this activation (Van den Bossche et al., 2017). Based on this concept, Rousseau et al. reduced fatty acid oxidation selectively in T cells by deleting the Peroxisome Proliferator-Activated Receptor beta/delta (PPARβ/δ). Deletion of PPARβ/δ in T cells increased the infiltration of T cells during skeletal muscle regeneration and prevented the age-induced decline in lean mass and endurance capacity. These effects are likely due to the inability of T cells to adjust their activation state. These three articles emphasize the role of resident and infiltrating immune cells in promoting skeletal muscle regeneration and maintenance.

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SKELETAL MUSCLE AS A SECRETORY ORGAN

Skeletal muscle is able to release "myokines" and small molecules to other types of cells and organs, with autocrine, paracrine, and long-distance endocrine effects. Bay and Pedersen discuss the role of skeletal muscle as a secretory organ, particularly regarding cytokines and growth factors acting on remote tissues such as adipose tissue, pancreas, liver, gut, and brain. Rogeri et al. focus on the role of glutamine and the myokine IL-6 in skeletal muscle and monocyte/macrophage functions. Under metabolic stress, such as exercise or an excess of fatty acids, skeletal muscle can also release small molecules such as ATP (Groen et al., 2019), a potent attractant and activator of immune cells (Pillon et al., 2014). Cruz and Beall demonstrate that extracellular ATP released by myotubes does not mediate fatty acid-induced insulin resistance but acts on myocytes to improve glucose uptake and glycolysis. These three studies illustrate the multiple inflammatory and metabolic roles of soluble molecules released by skeletal muscle.

SKELETAL MUSCLE ATROPHY

Loss of skeletal muscle mass has major health consequences, from decreased immunity to a higher risk of falls and fractures, leading to an increase in functional dependency and mortality (Marzetti et al., 2017). Muscle atrophy involves multiple factors including protein degradation by the ubiquitin-proteasome system (UPS). Tortola et al. reveal new regulators of the E3 ubiquitin ligase TRIM63 (MuRF1), which plays essential roles in UPS-mediated muscle atrophy. Using overexpression systems they propose the involvement of the transcription factor TFE3, protein kinase D (PKD2/3), and HDAC isoforms (HDAC-4 and HDAC-7). To promote skeletal muscle mass, Hagg et al. suggest a strategy to target the transmembrane prostate androgen-induced (TMEPAI) which inhibits the SMAD2/3 pathway. In mice, overexpression of TMEPAI increases skeletal muscle mass by as much as 30% and prevents muscle atrophy in a rodent model of cancer cachexia. With the same objective to prevent atrophy, Shen et al. find that the flavonoid isoquercitrin reduces inflammation, oxidative stress, UPS, and mitophagy, and overall protects against denervation-induced muscle mass loss. These three studies add to the current understanding of the molecular mechanisms underlying skeletal muscle atrophy.

SKELETAL MUSCLE ADAPTATION TO EXERCISE

Optimized exercise protocols to promote muscle force and hypertrophy have a wide range of applications, from improving performance in athletes to preventing metabolic diseases and cachexia, or delaying aging-associated sarcopenia (Cartee et al., 2016). Peake et al. investigate the effects of cold water immersion on the genes and proteins regulating muscle hypertrophy following an acute bout of resistance exercise. Their findings show that post-exercise cold water immersion can blunt muscle hypertrophy irrespective of exercise-induced alterations in factors that control skeletal muscle myogenesis, proteolysis, and extracellular matrix remodeling. Although this study did not directly look at this, it is plausible that cold water immersion would affect inflammatory responses and consequently impair skeletal muscle response to exercise (Tipton et al., 2017). Resistance exercise increases skeletal muscle inflammation, and macrophages play a critical role in the repair of skeletal muscle tissue in response to inflammation. However, in aged skeletal muscle, this tissue repair appears dysfunctional. Jensen et al. provide a 7-day time course of muscle macrophage activity and the response of downstream molecular targets following a single bout of resistance exercise. They observe a trend toward greater macrophage content in muscle biopsies from the elderly, and their findings further reveal that classically defined pro- and anti-inflammatory macrophage subtypes do not appear to exist in healthy aged skeletal muscle.

SKELETAL MUSCLE IN CANCER CACHEXIA

Cachexia is characterized by extreme weight loss, muscle wasting, systemic inflammation, and severe metabolic dysregulation (Argilés et al., 2018). Webster et al. describe pro-inflammatory cytokines and cellular processes associated with cachexia and their possible contribution to skeletal muscle atrophy. Focusing on the skeletal muscle microenvironment, VanderVeen et al. provide insights into the integrated networks of responses between immune cells, satellite cells, fibroblast cells, and endothelial cells and their regulatory role on myofiber size and plasticity. In mice, VanderVeen et al. demonstrate that the chemotherapy drug 5-fluorouracil can contribute to muscle wasting by depleting skeletal muscle immune cell populations. They demonstrate that infiltrating and resident immune cells in skeletal muscle are disrupted due to a sensitivity of skeletal muscle to the off-target effects of 5-fluorouracil.

Physical inactivity is commonly associated with cancer and contributes to muscle wasting. Yamada et al. describe that cancer-induced and inactivity-induced muscle atrophy are regulated by different mechanisms. In a preclinical mouse model of cancer cachexia, cancer exacerbated muscle wasting in denervated skeletal muscles, due to selective myosin loss, increased autophagy, and decreased protein synthesis. On the opposite, Leal et al. review the benefits of exercise training in cancer cachexia. Cellular and biochemical mechanisms by which exercise may counter cancer cachexia are discussed, as well as the challenges to the application of exercise protocols in clinical practice. These articles provide insights into the inflammatory state of skeletal muscle during cancer cachexia and the role of exercise as a countermeasure to prevent muscle mass loss.

SKELETAL MUSCLE IN METABOLIC DISEASES

Obesity and type 2 diabetes are associated with a chronic state of inflammation. Under metabolic stress, activated immune cells infiltrate the adipose, liver, and skeletal muscle tissues,

a mechanism contributing to the development of insulin resistance (Hotamisligil, 2017). In a transcriptomic meta-analysis, Manti et al. compare the signature of skeletal muscle in women with obesity and polycystic ovary syndrome (PCOS), a condition associated with metabolic dysfunction in women of reproductive age. They find a negative enrichment in inflammatory pathways, suggesting impaired immune function in skeletal muscles from women with PCOS. Both obesity and PCOS are associated with insulin resistance, which highlight the context-dependent ambivalent roles of the immune system on whole-body metabolism.

PERSPECTIVES

Inflammation is a key element of skeletal muscle adaptation to pathophysiological stresses, and which involves cellular (pro- and anti-inflammatory monocyte/macrophages, dendritic cells, T cells), and molecular actors (IL-6, TNFα, TGFβ, and TWEAK) that largely depend on whole-body homeostasis. An appropriate response involves an adequate and timely expression of inflammatory molecules (Chazaud, 2016). Elevated/uncontrolled inflammation leads to deleterious skeletal muscle adaptations and contributes to sarcopenia, cachexia, and metabolic diseases. This is also the case in several muscular dystrophies where inflammation, fibrosis, and/or muscle atrophy are major complications, often due to continuous muscle fiber breakdown (Serrano and Muñoz-Cánoves, 2017). In the context of muscular dystrophies, future directions will have to include combined approaches to holistically treat the primary genetic cause but also these secondary consequences (Cordova et al., 2018). More generally, future directions to finely tune muscle inflammation should not only include local pro- or anti-inflammatory strategies but should also consider holistic approaches to improve the overall skeletal muscle homeostasis through exercise, nutrition, as well as regulation of the immune system and metabolism. Future studies are needed to further understand the skeletal muscle immunometabolic signature in each of these contexts.

AUTHOR CONTRIBUTIONS

CT, AC, LS, MB, and NP contributed to the writing and editing of the manuscript. NP organized the work and finalized the manuscript. This manuscript has been approved by all named authors.

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REVIEW
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Divergent Roles of Inflammation in Skeletal Muscle Recovery From Injury

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A transient increase in local pro-inflammatory cytokine expression following skeletal muscle injury mediates the repair and regeneration of damaged myofibers through myogenesis. Regenerative capacity is diminished and muscle wasting occurs, however, when intramuscular inflammatory signaling is exceedingly high or persists chronically. An excessive and persistent inflammatory response to muscle injury may therefore impair recovery by limiting the repair of damaged tissue and triggering muscle atrophy. The concentration-dependent activation of different downstream signaling pathways by several pro-inflammatory cytokines in cell and animal models support these opposing roles of post-injury inflammation. Understanding these molecular pathways is essential in developing therapeutic strategies to attenuate excessive inflammation and accelerate functional recovery and muscle mass accretion following muscle damage. This is especially relevant given the observation that basal levels of intramuscular inflammation and the inflammatory response to muscle damage are not uniform across all populations, suggesting certain individuals may be more susceptible to an excessive inflammatory response to injury that limits recovery. This narrative review explores the opposing roles of intramuscular inflammation in muscle regeneration and muscle protein turnover. Factors contributing to an exceedingly high inflammatory response to damage and age-related impairments in regenerative capacity are also considered.

Keywords: myogenesis, satellite cells, myogenic regulatory factors, muscle protein turnover, cytokines

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INTRODUCTION

Skeletal muscle is the most abundant tissue in the human body, with a vital role in energy and protein metabolism as well as force generation for locomotion and stability. These essential functions can be disrupted with muscle injury occurring as a result of strains, contusions, lacerations, ischemia, burns, or even strenuous exercise. Musculoskeletal injuries requiring surgical interventions may also cause extensive muscle trauma. Damage to the muscle surrounding the hip or knee, for example, is inevitable during total hip or total knee arthroplasty (THA or TKA) procedures, respectively (Agten et al., 2017). Using a tourniquet to maintain a bloodless field

during surgery promotes muscle trauma through the associated ischemia and reperfusion injury (Muyskens et al., 2016). Loss of muscle mass or function due to injury may ultimately limit activities of daily living and quality of life.

Damaged skeletal muscle has the intrinsic capacity to regenerate and repair itself through myogenesis. The myogenic response involves activation, proliferation, and differentiation of muscle-resident stem cells (i.e., satellite cells) that ultimately fuse with each other and existing fibers to restore injured tissue (Dumont et al., 2015; Snijders et al., 2015). Muscle regenerative capacity is diminished with advanced age and with chronic inflammation (Suetta et al., 2013; Jin et al., 2018). Intramuscular inflammatory signaling plays a critical role in mediating the regenerative response to muscle fiber damage and must be finely regulated given inflammatory cytokine expression is capable of promoting muscle growth and muscle loss (Dogra et al., 2006, 2007; Munoz-Canoves et al., 2013). A transient increase in local inflammatory signaling triggers a pro-myogenic signaling cascade that aids in the repair, remodeling, and maintenance of healthy muscle tissue. If this intramuscular inflammation persists chronically, however, regenerative capacity is diminished and muscle atrophy ensues. An exceedingly high inflammatory response to muscle damage would limit recovery by preventing the repair of damaged tissue, promoting muscle wasting, and ultimately impairing the restoration of muscle function.

This narrative review explores recent advances delineating the opposing roles of intramuscular inflammation in regeneration, maintenance, and wasting of muscle. Intrinsic and extrinsic factors underlying the loss of regenerative capacity with aging are also discussed. Understanding molecular and cellular processes regulating muscle regeneration versus muscle atrophy downstream of inflammatory signaling, as well as age-related changes in these pathways, is essential in developing therapeutic strategies to attenuate excessive inflammation, restore regenerative capacity, and accelerate recovery following muscle injury. Identifying individuals susceptible to excessive muscle inflammation has also been proposed as a possible method of predicting muscle mass and functional recovery potential following orthopedic injury (Bamman et al., 2015). The utility of this concept in clinical settings is therefore considered.

Abbreviations: 4E-BP1, 4E binding protein 1; ACL, anterior cruciate ligament; AMPK, AMP-activated protein kinase; CCL2, CC-chemokine ligand 2; CXCL1, CXC-chemokine ligand 1; DAMP, damage-associated molecular pattern; Dll4, Delta-like 4; ECM, extra-cellular matrix; eIF2B, eukaryotic initiation factor 2B; FAP, fibro-adipogenic progenitor; FGF-2, fibroblast growth factor-2; Fn14, fibroblast growth factor-inducible 14; FOXO, forkhead box O; GSK-3β, glycogen synthase kinase 3β; HMGB1, high-mobility group box 1 protein; IFNγ, interferon gamma; IGF-1, insulin-like growth factor 1; IL, interleukin; IRS-1, insulin receptor substrate 1; JAK, Janus kinase; MAFbx, muscle atrophy F-box; MRF, myogenic regulatory factors; MRF4, myogenic regulatory factor 4; mTOR, mammalian target of rapamycin; MuIS, muscle inflammatory susceptibility; MuRF1, muscle ring finger 1; Myf5, myogenic factor 5; NF-κB, nuclear factor-κB; p38 MAPK, p38 mitogen-activated protein kinase; p70S6K, p70 ribosomal protein S6 kinase; Pax7, paired box 7; PI3K, phosphoinositide 3-kinase; RBP-Jk, recombining protein-Jk; STAT3, signal transducer and activator of transcription 3; Spry1, Sprouty1; TGF-β1, transforming growth factor beta 1; THA, total hip arthroplasty; TKA, total knee arthroplasty; TNFα, tumor necrosis factor-α; T_{reg}, regulatory T cell; TWEAK, TNF-like weak inducer of apoptosis; Ulk1, Unc-51-like kinase 1; UPS, ubiquitin proteasome system.

SKELETAL MUSCLE REGENERATION

Satellite Cells and Myogenesis

Skeletal muscle regeneration after injury involves the activation of muscle-resident satellite cells located outside the sarcolemma and underneath the basal lamina of muscle fibers (Mauro, 1961). These normally quiescent adult muscle stem cells are regulated by a dynamic interaction of extrinsic factors [i.e., systemic molecules, fibro-adipogenic progenitors (FAP), immune cells] and intracellular signaling pathways that mediate satellite cell quiescence, self-renewal, and the myogenic response (Li, 2003; Girgenrath et al., 2006; Arnold et al., 2007; Fiore et al., 2016). Activated satellite cells generate myoblasts in response to injury-related signals by reentering the cell cycle and proliferating. Myoblasts subsequently differentiate by increasing expression of muscle-specific genes, and eventually fuse with each other or existing myofibers to regenerate and repair damaged tissue. A small subset of activated satellite cells do not commit to myogenic differentiation and instead replenish the satellite cell pool by returning to a quiescent state (Olguin and Olwin, 2004; Zammit et al., 2004).

Satellite cell progression through the myogenic program is driven by the sequential expression of specific transcription factors. Pax7 is a paired box transcription factor that is expressed in all satellite cells of mature muscle and is critical to their function (Seale et al., 2000; von Maltzahn et al., 2013). Terminal differentiation requires down-regulation of Pax7, while elevation of Pax7 subsequent to proliferation promotes a return to quiescence (Olguin and Olwin, 2004; Olguin et al., 2007). Pax7 has also been implicated in satellite cell specification of pluripotent stem cells, as it appears to restrict alternate development programs (Seale et al., 2000). Progression of myogensis also involves Myf5, MyoD, myogenin, and MRF4, transcription factors collectively known as Myogenic Regulatory Factors (MRFs). Myf5 and MyoD are expressed early after injury (Cornelison and Wold, 1997; Cooper et al., 1999), while myogenin and MRF4 have been shown to regulate later stages of myogenic differentiation (Smith et al., 1994; Cornelison and Wold, 1997).

A precise balance of intracellular mechanisms regulating quiescence, differentiation, and self-renewal are necessary to maintain satellite cell function and regenerative capacity. Notch signaling, for example, is critical to multiple cellular processes. Injury-related activation of Notch pathway activity stimulates satellite cell proliferation and inhibits differentiation, allowing for adequate expansion of progenitor cells (Conboy and Rando, 2002). Eventual suppression of this pathway and transition to canonical Wnt signaling appears necessary for proper differentiation (Brack et al., 2008). Notch signaling also plays a role in maintaining satellite cell quiescence and self-renewal capacity. Specific deletion of the Notch signaling protein recombining protein-Jk (RBP-Jk) triggered spontaneous satellite cell activation and attenuated self-renewal, ultimately depleting the satellite cell pool (Bjornson et al., 2012). Low et al. (2018) have also shown that activation of Notch3 by the Notch ligand Delta-like 4 (Dll4) during muscle repair provided a signal for

satellite cells to exit the cell cycle and return to quiescence, thereby promoting self-renewal. FOXO3 expression and Sprouty 1 (Spry1) activity have similarly been implicated in the return of satellite cells to quiescence during repair to maintain the satellite cell pool (Shea et al., 2010; Gopinath et al., 2014).

Skeletal muscle autophagy, a cellular process responsible for the degradation of intracellular proteins, macromolecules, and organelles, is critical for skeletal muscle regeneration. Deletion of the autophagy initiation kinase Unc-51-like kinase 1 (Ulk1) in mice subjected to muscle injury impaired recovery of muscle strength and mitochondrial protein content (Call et al., 2017). Age-related declines in autophagic activity are similarly associated with diminished regenerative capacity (Garcia-Prat et al., 2016). Autophagy appears necessary to support rapid changes in metabolic activity and bioenergetic demands of activated versus quiescent satellite cells (Tang and Rando, 2014). Inhibiting autophagy in satellite cells suppressed ATP production and delayed their activation (Tang and Rando, 2014). Myoblast differentiation and fusion capacity were similarly impaired with inhibition of autophagy (McMillan and Quadrilatero, 2014). AMP-activated protein kinase (AMPK)-mediated phosphorylation of $p27^{Kip1}$ and its subsequent translocation from the nuclei to the cytoplasm has been implicated in the regulation of autophagy during regeneration (White et al., 2018). In total, these catabolic mechanisms are imperative to the regenerative process.

Inflammatory Response to Skeletal Muscle Injury

A highly controlled, time-dependent activation of immune cells occurs rapidly after muscle injury to remove necrotic tissue and release soluble factors that regulate satellite cell activation and progression through the differentiation process (Tidball, 2017; Yang and Hu, 2018). Acute pro-inflammatory signaling and immune cell recruitment to the site of muscle trauma constitutes the initial phase of this inflammatory response. Immune cell infiltration at the site of injury is first stimulated by the complement system, which is activated immediately following muscle damage (Frenette et al., 2000). Injury-related activation of muscle-resident mast cells and their subsequent degranulation and release of inflammatory mediators (i.e., TNFα, IL-1, histamine) similarly promotes immune cell recruitment (Gorospe et al., 1996; Radley and Grounds, 2006). Secretion of the chemoattractants CXC-chemokine ligand 1 (CXCL1) and CC-chemokine ligand 2 (CCL2) as well as damage-associated molecular patterns (DAMPs) such as high-mobility group box 1 protein (HMGB1) from muscle-resident macrophages also facilitates immune cell activation and infiltration following injury (Brigitte et al., 2010; Venereau et al., 2012). Recent work has suggested muscle osteopontin expression is also involved in promoting immune cell infiltration (Wasgewatte Wijesinghe et al., 2019).

Several immune cells are involved in the skeletal muscle regenerative response. Neutrophils are among the first inflammatory cells to infiltrate injured muscle tissue. They are observed as early as 1 – 3 h after muscle damage, with maximum numbers seen 6 – 24 h after injury (Fielding et al., 1993; Malm et al., 2000). These phagocytic cells release proteases and oxidants to facilitate the removal of damaged fibers. Although their presence can aggravate muscle injury (Pizza et al., 2005), neutrophil depletion delays muscle recovery, suggesting their role in clearing cellular debris and recruiting other inflammatory cells is critical for successful regeneration (Teixeira et al., 2003). Recent work also indicates that neutrophils may have anti-inflammatory and inflammation-resolving properties important to the progression of later stages of myogenesis (Sugimoto et al., 2016).

The temporal and spatial recruitment of macrophages is critical to the muscle regenerative response to injury. Neutrophil production of interleukin-1 (IL-1) and interleukin-8 (IL-8) at the site of injury, and the secretion of CCL2 from injured muscle fibers and resident macrophages stimulates the extravasation of monocytes into injured tissue and their subsequent differentiation into macrophages (Fujishima et al., 1993; Lu et al., 2011). Inhibiting this accumulation of monocytes and macrophages in injured muscle using $Ccr2^{-/-}$ mice and via pharmacological treatment (i.e., liposomal clodronate administration) impairs the regenerative response (Summan et al., 2006; Lu et al., 2011). Autophagy and caspase activation have also been implicated in this monocyte to macrophage transition (Jacquel et al., 2012). Macrophages are initially polarized into a pro-inflammatory M1 phenotype that secretes pro-inflammatory cytokines (i.e., TNFα, IL-1β, IFN-γ) and reactive oxygen species to facilitate the removal of cellular debris and recruitment of immune cells to the lesion site (Villalta et al., 2009; Dort et al., 2019). This early increase in M1 cells precedes the eventual expansion of anti-inflammatory M2 macrophages that repress the local inflammatory response and promote muscle growth. M1 macrophages reach peak numbers 1–2 days after injury, while M2 cells are the predominant macrophage population by 3 days post-injury (Arnold et al., 2007).

Although a mixture of M1 and M2 cells have been observed following injury (Heredia et al., 2013), a switch in the overall bias of macrophage populations from a pro-inflammatory to anti-inflammatory phenotype is critical for the proper progression of the regenerative response. Pro-inflammatory cytokines released from M1 macrophages stimulate myoblast proliferation, while anti-inflammatory cytokines from M2 macrophages promote their differentiation (Arnold et al., 2007). Suppressing this M1 to M2 phenotype transition has been shown to attenuate muscle regeneration and reduce muscle fiber growth (Mounier et al., 2013; Tonkin et al., 2015; Varga et al., 2016). Several molecular processes regulate the ability of macrophages to switch from the M1 to M2 phenotype. Phagocytosis of apoptotic cells by activated macrophages, for example, attenuates macrophage pro-inflammatory cytokine production and enhances their anti-inflammatory activity (Chung et al., 2006). Increased concentrations of IL-10 similarly promote the M2 phenotype (Villalta et al., 2011b), while interferon gamma (IFNγ) signaling attenuates the M1 to M2 conversion (Villalta et al., 2011a). Recent work has also implicated the heme-binding transcriptional repressor BACH1 in this response, as *Bach1* knockout mice displayed impairments

in muscle regeneration and the macrophage phenotype switch (Patsalos et al., 2019).

T lymphocyte (T cell) recruitment is also essential in the repair and regeneration of damaged muscle tissue, with infiltration of $CD4^+$ and $CD8^+$ T cells peaking around 3–5 days post-injury (Fu et al., 2015). T cell-deficient *Rag1*$^{-/-}$ mice have delayed muscle regeneration after injury that is reversed with the transplantation of active $CD3^+$ T cells (i.e., general population of activated T cells). Muscle regeneration capacity is similarly attenuated in $CD8^+$-deficient mice (Zhang et al., 2014) and with the depletion of $Foxp3^+$ $CD4^+$ regulatory T cells (T_{reg} cells) (Burzyn et al., 2013). T_{reg} cells appear to regulate muscle regeneration by acting directly on satellite cells and modulating immune cell activity. Accumulation of T_{reg} cells occurs at a time when infiltrating macrophages switch from a M1 pro-inflammatory to M2 pro-regenerative state, and deletion of T_{reg} cells impairs this phenotypic transition and prolongs the inflammatory response (Burzyn et al., 2013). Muscle T_{reg} cells also release amphiregulin, a ligand for the epidermal growth factor receptor that enhances satellite cell differentiation *in vitro* and *in vivo* (Burzyn et al., 2013).

Transient remodeling of extra-cellular matrix (ECM) components and modulation of satellite cell activity by FAP cells is an additional component to the muscle regenerative response to injury. FAPs are stem cells that can differentiate into fibroblasts or adipocytes. They are normally quiescent in healthy muscle, but proliferate rapidly in response to muscle injury (Joe et al., 2010). Depletion of FAPs impairs muscle regeneration after injury and leads to a loss of satellite cells under homeostatic conditions (Wosczyna et al., 2019). FAPs appear to regulate the regenerative response by influencing satellite cell activity. Pharmacological blockage of FAP expansion prevented transient ECM deposition and attenuated myoblast proliferation *in vivo* (Fiore et al., 2016). *In vitro* work similarly demonstrated a dose-dependent increase in myoblast proliferation with the addition of proliferating FAPs to satellite cell cultures (Fiore et al., 2016). This work collectively highlights the critical role of immune cell infiltration, ECM remodeling, and inflammatory signaling in regulating muscle regeneration after injury.

POTENTIAL CONSEQUENCES OF HEIGHTENED INTRAMUSCULAR INFLAMMATORY SIGNALING

The release of inflammatory cytokines following muscle damage is a finely regulated response. Several pro-inflammatory cytokines (e.g., TNFα, TWEAK) involved in initial stages of muscle regeneration and repair are elevated early in response to damage (Li, 2003; Girgenrath et al., 2006). A shift from pro- to anti-inflammatory signaling (e.g., IL-13, IL-10, IL-4) within days of muscle injury subsequently represses local inflammatory signaling and supports later phases of myogenesis (Arnold et al., 2007; Deng et al., 2012). Heightened basal (pre-injury or pre-surgery) levels of intramuscular inflammation, an exaggerated inflammatory response to muscle damage, or both, would lead to exceedingly high levels of muscle inflammation that may disrupt this finely regulated response. Likewise, failure to resolve persistent pro-inflammatory signaling after muscle injury may dysregulate muscle regeneration and repair.

Exceedingly high or unresolved post-injury inflammation would impact many aspects of the regenerative response. Chronic infection of mouse skeletal muscle (i.e., a preexisting inflammatory environment) delays muscle repair following cardiotoxin injury (Jin et al., 2018). The heightened inflammatory environment of infected muscle appeared to limit macrophage phenotype transition and altered the expression of MRFs following damage (Jin et al., 2018). A delayed switch from pro-inflammatory to pro-regenerative macrophages may also promote fibrosis. An overlap in expression of M1- and M2-derived tumor necrosis factor-α (TNFα) and transforming growth factor beta 1 (TGF-β1), respectively, has been shown to reduce FAP apoptosis and increase ECM deposition (Lemos et al., 2015). Expression of the TNFα receptors TNFR1 and TNFR2 in nerve structures during chronic inflammatory insult in rabbit muscle suggests the consequences of excessive inflammation extend beyond the muscular system (Renstrom et al., 2017).

Persistent pro-inflammatory cytokine signaling may promote muscle wasting by blunting muscle protein synthesis and triggering muscle protein breakdown. This is evident with several pathophysiological conditions of muscle wasting. Burn injury, as an extreme example, leads to skeletal muscle breakdown throughout the body. A marked increase in vastus lateralis mRNA expression of TNFα-, TNF-like weak inducer of apoptosis (TWEAK)- and interleukin (IL)-6-family cytokines and receptors 5-days post burn injury suggests changes in inflammatory signaling may contribute to the observed atrophy (Merritt et al., 2013b). Similarly, elevated expression of TWEAK and TNFα receptors has been observed in paralyzed muscle of men with chronic spinal cord injury, a condition that also presents with muscle atrophy and impaired regenerative capacity (Yarar-Fisher et al., 2016). The opposing regulatory roles of local inflammatory signaling in myogenesis and muscle mass maintenance indicate the post-injury or post-operative inflammatory response must be well-controlled to maintain muscle mass and allow for adequate tissue regeneration. While multiple cytokines are involved in regulating these processes, TWEAK, TNFα, IL-6 and their respective molecular pathways appear to be shared across conditions of both acute and chronic inflammation.

Divergent Roles of Inflammation in Myogenesis

The opposing roles of inflammation in promoting and inhibiting myogenesis correspond with observed concentration- and time-dependent effects of several inflammatory cytokines in cell and animal models (**Figure 1**). TWEAK, for example, is a cytokine produced by several cell types (i.e., macrophages and skeletal muscle) that is capable of modulating myogenesis (Mittal et al., 2010). Initial studies demonstrated that high concentrations of exogenous TWEAK (≥100 ng/mL) in cultured myoblasts enhanced proliferation but inhibited subsequent differentiation and myotube formation (Dogra et al., 2006; Girgenrath et al., 2006). The observed impairment in cell cycle exit and

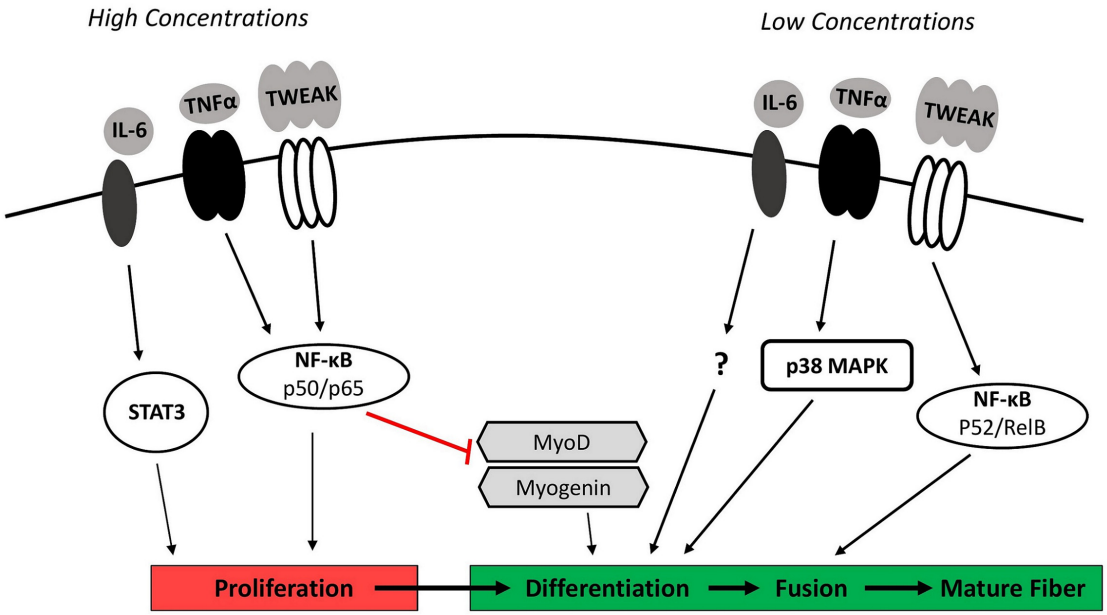

FIGURE 1 | Inflammation and myogenesis. While multiple cytokines are involved in coordinating the myogenic response to injury, major regulators include IL-6, TNFα, and TWEAK. High concentrations of these cytokines signaling via STAT3 and the classical NF-κB pathway (p50/p65 heterodimer) stimulate myoblast proliferation but inhibit subsequent differentiation through the degradation and destabilization of the myogenic regulatory factors MyoD and myogenin. Low concentrations of IL-6, TNFα, and TWEAK signaling through p38MAPK and the alternative NF-κB pathway (p52/RelB heterodimer) are necessary to facilitate later stages of myogenesis by stimulating myoblast differentiation and fusion. IL-6, interleukin-6; NF-κB, nuclear factor-κB; p38 MAPK, p38 mitogen-activated protein kinase; STAT3, signal transducer and activator of transcription 3; TNFα, tumor necrosis factor-α; TWEAK, TNF-like weak inducer of apoptosis.

muscle specific gene expression in TWEAK-treated myoblasts corresponded with decreased gene expression and protein levels of the myogenic regulatory factors MyoD and myogenin (Dogra et al., 2006; Girgenrath et al., 2006). Activation of classical (canonical) nuclear factor-kB (NF-κB) signaling appears to mediate this response. NF-κB proteins are a family of structurally similar transcription factors (p65, RelB, c-Rel, p105/p50, and p100/p52) that form either homodimers or heterodimers. Classical signaling specifically involves the phosphorylation and degradation of an inhibitory protein (IκBα) and subsequent translocation of an activated p65/p50 heterodimer to the nucleus (Bakkar and Guttridge, 2010). Inhibiting p65/p50 activity reversed the inhibitory effect of soluble TWEAK (500 ng/mL) on differentiation and MyoD protein expression in cultured myoblasts (Dogra et al., 2006), suggesting high concentrations of TWEAK limit normal myogenic progression through classical NF-κB activity.

High concentrations of TWEAK enhancing proliferation and inhibiting differentiation through classical NF-κB signaling would be critical during early phases of muscle regeneration to promote rapid proliferation and adequate expansion of the myoblast population, while simultaneously avoiding premature differentiation (Enwere et al., 2014). In contrast, TWEAK present at low concentrations (10 ng/mL) in C2C12 myoblasts has been shown to promote later stages of myogenesis through the activation of alternative (non-canonical) NF-κB signaling (Enwere et al., 2012). The alternative NF-κB signaling pathway involves the phosphorylation and partial proteasomal degradation of a p100 subunit to p52 to generate a p52/RelB heterodimer (Bakkar and Guttridge, 2010). Translocation of the p52/RelB heterodimer to the nucleus and subsequent transcriptional activity promotes myogenesis by stimulating myoblast fusion into myotubes. Exposing differentiating myoblasts to low concentrations of exogenous TWEAK (10 ng/mL) increased myoblast fusion and doubled myotube diameter when compared to untreated cells (Enwere et al., 2012).

The cytokine TNFα also regulates myogenesis. TNFα concentrations rise substantially at the site of muscle injury due to its release from injured myofibers and infiltrating immune cells (De Bleecker et al., 1999). *In vitro* work has shown that TNFα intrinsic to satellite cells and myeloid cell-derived TNFα influence the myogenic response (Wang et al., 2018). The early increase in TNFα expression functions as a chemoattractant signal that stimulates myogenic cell migration to the site of injury (Torrente et al., 2003). The initially high concentrations of TNFα also play a role in promoting proliferation (Li, 2003) and inhibiting myogenic differentiation (Langen et al., 2004). This occurs analogous to TWEAK through the activation of classical NF-κB signaling and downstream inhibition of MyoD mRNA and protein expression (Langen et al., 2004). An abundance of myeloid cell-derived TNFα also appears to reduce muscle cell fusion (Wang et al., 2018). While high concentrations of recombinant TNFα (≥0.5 ng/mL) inhibit the progression of myogenesis, low concentrations of TNFα (0.05 ng/mL) have been shown to enhance differentiation in cultured myoblasts (Chen et al., 2007). This response appears to occur through

downstream activation of p38 mitogen-activated protein kinase (MAPK). Levels of activated p38 MAPK and markers of differentiation were both diminished when TNFα was neutralized in C2C12 myoblast (Chen et al., 2007). p38 activation and muscle regeneration were also impaired following cardiotoxin-injury of soleus muscle in TNFα receptor double knockout mice ($p55^{-/-}p75^{-/-}$) (Chen et al., 2005).

The cytokine IL-6 is another major regulator of myogenesis. IL-6 is secreted by infiltrating macrophages and neutrophils, FAP cells, and muscle itself (Kami and Senba, 1998; Joe et al., 2010; Zhang et al., 2013). Binding of IL-6 to its receptor activates the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling cascade. IL-6-dependent activation of STAT3 specifically was required for satellite cell proliferation *in vitro* (Serrano et al., 2008). IL-6 is also necessary for the complete differentiation of muscle cells. Primary muscle cells from $IL\text{-}6^{-/-}$ mice displayed a clear reduction in myotube formation indicative of decreased myoblast fusion (Hoene et al., 2013). This occurred independent of downstream STAT3 activation, although the exact mediators of this effect are unknown. How exceedingly high levels of IL-6 affect myogenesis is unclear, however, chronic overexpression of IL-6 has been shown to induce muscle wasting (Haddad et al., 2005).

In total, these findings specific to TWEAK, TNFα, and IL-6 indicate that high concentrations of these cytokines may inhibit normal myogenic progression. It is important to note, however, that the physiological relevance of these *in vitro* studies remains unclear as they generally involve acute exposure to very high concentrations of a single cytokine. The involvement of multiple cytokines under physiological conditions and the varied concentrations of cytokines between plasma and muscle interstitium due to the presence of an endothelial barrier are possible confounding factors that must be considered. Regardless, these findings collectively suggest that a transient increase in intramuscular pro-inflammatory signaling after injury is required for muscle regeneration and repair, while an excessive or persistent inflammatory response can prevent myogenesis and limit recovery.

Inflammation Modulates Muscle Protein Turnover

Exceedingly high or chronic expression of pro-inflammatory cytokines following muscle injury impairs recovery by promoting muscle wasting (**Figure 2**). Differentiated C2C12 myotubes incubated with soluble TWEAK (10 ng/mL), for example, display reduced mass and a loss of total protein content (Dogra et al., 2007). Mice subjected to chronic administration of soluble TWEAK similarly exhibit reduced fiber diameter in isolated muscle sections and decreased body weight compared to control mice (Dogra et al., 2007). This effect appears to involve multiple signaling pathways that promote protein catabolism and impair anabolic signaling. Activation of NF-κB, for example, is involved in the TWEAK-induced degradation of cultured myotubes (Dogra et al., 2007). NF-κB signaling likely mediates this effect by up-regulating the ubiquitin proteasome system (UPS). The UPS is largely responsible for the degradation of myofibrillar proteins through the enzymatic activity of the muscle-specific ubiquitin ligases muscle atrophy F-box (MAFbx/Atrogin-1) and muscle ring finger 1 (MuRF1). NF-κB has been shown to regulate MuRF1 expression (Cai et al., 2004), which was increased along with MAFbx following TWEAK treatment of cultured myotubes (Dogra et al., 2007).

Inhibition of the phosphatidylinositol-3 kinase (PI3K)/Akt pathway also contributes to the observed up-regulation of UPS activity in TWEAK-treated myotubes. Active Akt phosphorylates and inhibits the forkhead box O (FoxO) transcription factor, preventing its translocation to the nucleus and induction of MAFbx and MuRF1 expression (Stitt et al., 2004). Exogenous TWEAK in cultured myotubes significantly decreased Akt and FOXO1a phosphorylation, suggesting increases in MAFbx and MuRF1 following TWEAK treatment are also due to Akt inhibition (Dogra et al., 2007). TWEAK-mediated inhibition of the PI3K/Akt is also consequential to anabolic signaling. Akt facilitates translation initiation and muscle protein synthesis through the phosphorylation and activation of the mammalian target of rapamycin (mTOR) and subsequent activity of its downstream targets, p70 ribosomal protein S6 kinase (p70S6K) and 4E binding protein 1 (4E-BP1) (Nave et al., 1999). In addition, active Akt phosphorylates glycogen synthase kinase 3β (GSK-3β), reversing its inhibitory effect on the translation initiation factor eukaryotic initiation factor 2B (eIF2B) (Jefferson et al., 1999). Phosphorylation levels of GSK-3β, mTOR, and p70S6K were decreased following TWEAK treatment of cultured myotubes, indicating impaired anabolic signaling (Dogra et al., 2007). An imbalance where catabolic signaling exceeds anabolic signaling promotes muscle wasting.

Elevated levels of TNFα modulate muscle protein turnover and induce atrophy. A dose-dependent loss of total protein was noted in differentiated skeletal muscle myotubes following prolonged exposure to TNFα (1 – 6 ng/mL) (Li and Reid, 2000). TNFα appears to mediate this effect by activating NF-κB transcriptional activity (Li and Reid, 2000) and inhibiting Akt (Sishi and Engelbrecht, 2011), triggering downstream up-regulation MAFbx and MuRF1 expression. The up-regulation of MAFbx in C2C12 myoblasts following TNFα exposure also required intact p38 MAPK, indicating the TNFα/p38 MAPK signaling axis enhances downstream UPS activity and related muscle catabolism (Li et al., 2005). Increased TNFα concentrations have also been shown to limit muscle anabolism via the PI3K/Akt signaling pathway. Upstream activation of PI3K/Akt signaling involves the binding of factors such as insulin-like growth factor 1 (IGF-1) or insulin to their receptors and the subsequent phosphorylation and activation of insulin receptor substrate 1 (IRS-1). TNFα may exert its anti-anabolic effect by downregulating IGF-1 synthesis or by direct interaction with IRS-1. Addition of TNFα to cultured myotubes decreased IGF-1 mRNA expression by 50–80% (Frost et al., 2003). TNFα has also been shown to stimulate serine residue phosphorylation of IRS-1, preventing its recruitment to the insulin/IGF-1 receptor (Hotamisligil et al., 1996). In theory, this would ultimately suppress anabolic (i.e., mTOR, p70S6K) and promote catabolic (i.e., FOXO, MuRF1, MAFbx) signaling downstream of the IGF-1 receptor.

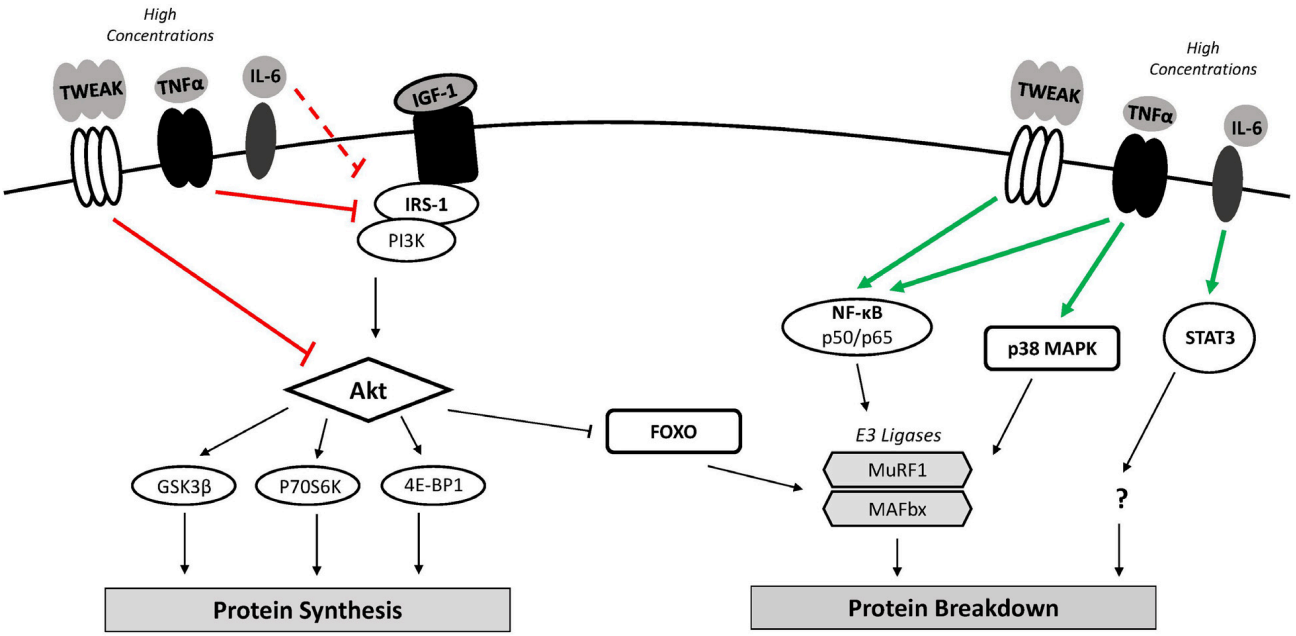

FIGURE 2 | Inflammation and muscle protein turnover. Classical muscle wasting cytokines include TWEAK, TNFα, and IL-6. High concentrations of these cytokines may down-regulate mRNA translation and muscle protein synthesis through inhibition of the PI3K/Akt pathway resulting in GSK3β inhibition of translation and reduced mTOR activation. Increased FOXO nuclear localization along with classical NF-κB and p38 MAPK activity increases transcription of atrophy-related genes (i.e., MuRF1, MAFbx). An imbalance where catabolic signaling exceeds anabolic signaling can lead to muscle atrophy. 4E-BP1, 4E binding protein 1; FOXO, forkhead box O; GSK-3β, glycogen synthase kinase 3β; IGF-1, insulin-like growth factor 1; IL-6, interleukin-6; IRS-1, insulin receptor substrate 1; MAFbx, muscle atrophy F-box; mTOR, mammalian target of rapamycin; MuRF1, muscle ring finger 1; NF-κB, nuclear factor-κB; p38 MAPK, p38 mitogen-activated protein kinase; p70S6K, p70 ribosomal protein S6 kinase; PI3K, phosphoinositide 3-kinase; STAT3, signal transducer and activator of transcription 3; TNFα, tumor necrosis factor-α; TWEAK, TNF-like weak inducer of apoptosis.

The effects of heightened IL-6 expression on muscle anabolic and catabolic signaling pathways *in vivo* remain elusive. Locally infusing soluble IL-6 in muscles of rats significantly decreased myofibrillar protein content compared to untreated contralateral muscle (Haddad et al., 2005). Inhibiting STAT3 in C2C12 myoblasts reduced muscle atrophy downstream of IL-6, implicating the JAK/STAT3 pathway as a mediator of IL-6-induced muscle wasting (Bonetto et al., 2012). The mechanisms by which STAT3 promotes atrophy, however, are still unknown. IL-6-mediated muscle wasting may also result from a downregulation of anabolic signaling pathways. An increase in IGF-1 mRNA and decrease in downstream phosphorylation of p70S6K has been observed in IL-6-infused muscles of rats (Haddad et al., 2005). These findings suggest that IL-6 disrupts growth factor-related intracellular signaling, resulting in a compensatory increase in IGF-1 production (Haddad et al., 2005) which would potentially compromise skeletal muscle mass.

VARIABILITY IN MUSCLE REGENERATIVE CAPACITY

Activation of satellite cells and their subsequent proliferation, differentiation, and fusion with existing myofibers is critical after muscle injury to regenerate damaged muscle tissue. However, age-related declines in progenitor cell expansion and inadequate maintenance of satellite cell quiescence impairs muscle regenerative capacity in older adults. Heterochronic parabiosis and whole muscle grafting experiments have shown that regeneration of old muscle exposed to a young environment occurs normally, implicating extrinsic factors and age-related alterations in the satellite cell microenvironment in the dysregulation of muscle regeneration with aging (Carlson and Faulkner, 1989; Conboy et al., 2005). However, recent work using isolated satellite cells has also associated cell-intrinsic defects with the age-related decline in satellite cell function suggesting regulation is multifaceted (Bernet et al., 2014; Cosgrove et al., 2014; Sousa-Victor et al., 2014).

Several molecular and cellular processes (both intrinsic and extrinsic) underlie to the loss of regenerative capacity with aging. Notch signaling in young muscle promotes proliferation of activated satellite cells, while a subsequent suppression of this pathway and transition to canonical Wnt signaling is necessary for proper differentiation and muscle growth (Conboy and Rando, 2002; Brack et al., 2008). Aging appears to disrupt the precise balance and interaction of these pathways, as Notch activity is attenuated in aged muscle (Conboy et al., 2003). Satellite cells isolated from aged mice also display high levels of Wnt signaling that attenuate progression of satellite cells through the differentiation program and promote their adoption of a

fibrogenic fate (Brack et al., 2007). Recent work has suggested age-related impairments in myogensis are also tied to declines in autophagy, which causes functional deterioration of satellite cells and promotes senescence (Garcia-Prat et al., 2016). Age-related perturbations in IL-33 expression may similarly disrupt regeneration by altering the accumulation of T_{reg} cells in the satellite cell niche (Kuswanto et al., 2016). Additional changes to the satellite cell microenvironment including increased fibroblast growth factor-2 (FGF-2) expression, loss of fibronectin, systemic increases in GDF11, impaired β1-Integrin signaling, and attenuated levels of the anti-aging hormone Klotho have all been implicated in the poor repair of skeletal muscle with aging (Chakkalakal et al., 2012; Egerman et al., 2015; Lukjanenko et al., 2016; Rozo et al., 2016; Sahu et al., 2018; Welc et al., 2020).

Age-related increases in intramuscular inflammatory pathway activity may also reduce muscle regenerative potential with aging. Animal work has indicated that attenuating IL-6/JAK/STAT3 pathway activity enhances regenerative potential of aged satellite cells (Price et al., 2014). Merritt et al. (2013a) also observed differences in basal muscle pro-inflammatory signaling independent of circulating cytokines between middle-aged adults (40.4 ± 1.1 y, AGE40), older adults (61.2 ± 0.6 y, AGE61), and elderly (75.5 ± 0.7 y, AGE76) individuals. IL-6, TNFα, and TWEAK expression were higher in AGE61 and AGE76 groups relative to AGE40 at baseline and following modest muscle damage induced with a resistance exercise protocol (Merritt et al., 2013a). Myoblasts isolated from three young (28 ± 2 y) versus three older (64 ± 2 y) individuals also displayed greater inflammatory signaling in the absence of a pro-inflammatory stimulus, and had an exaggerated inflammatory response and reduced fusion capacity when treated with TNFα (Merritt et al., 2013a). This heightened basal pro-inflammatory signaling and hypersensitivity to inflammatory stimuli in individuals of advanced age was described as "muscle inflammation susceptibility," or MuIS$^{(+)}$. This MuIS$^{(+)}$ phenotype, along with age-related cell-intrinsic and extrinsic defects affecting satellite cell self-renewal (Bernet et al., 2014), may impair myogenesis and promote muscle atrophy following muscle injury.

Basal levels of inflammation and the sensitivity to inflammatory stimuli may not be uniform across all populations. Follow-up work by Bamman et al. (2015) described analyses identifying the MuIS$^{(+)}$ phenotype in certain adults across all ages, suggesting some individuals display elevated basal levels of muscle inflammation and an exaggerated or prolonged response to an inflammatory insult. This apparent interindividual variability in heightened inflammation susceptibility suggests some individuals may be more prone to an exceedingly high inflammatory response to muscle injury that would be detrimental to recovery. Bamman et al. (2015) considered this hypothesis by evaluating MuIS status in patients undergoing total hip arthroplasty (THA) for end-stage osteoarthritis, since osteoarthritis-related damage in the hip leads to immune cell infiltration and heightened inflammatory cytokine expression in the synovial fluid, synovial membrane, cartilage, and the subchondral bone layer (Wojdasiewicz et al., 2014; Mathiessen and Conaghan, 2017). In theory, evaluating MuIS status would ascertain the susceptibility of muscle surrounding the hip to the local inflammatory burden of the osteoarthritic joint (Bamman et al., 2015).

The MuIS$^{(+)}$ phenotype was identified in a population of THA patients based on the expression of the TWEAK receptor, fibroblast growth factor inducible 14 (Fn14), in muscle surrounding the diseased hip (Bamman et al., 2015). Levels of Fn14 are generally low in healthy tissues and therefore the induction of Fn14 expression in response to injury, stress, or exercise is tied to TWEAK/Fn14 pathway activity (Enwere et al., 2014). Dichotomization into MuIS$^{(+)}$ and MuIS$^{(-)}$ individuals was done based on median Fn14 expression and revealed several key differences between groups. The MuIS$^{(+)}$ group (n = 7) had a mean Fn14 gene expression that was five times higher than the MuIS$^{(-)}$ group (n = 8) (Bamman et al., 2015). Individuals designated as MuIS$^{(+)}$ also exhibited heightened expression of all inflammatory genes evaluated (e.g., TNFα, IL-6, TWEAK) compared to non-surgical controls, while only the up-regulation of the IL-6 receptor (IL-6R) was found in the MuIS$^{(-)}$ group (Bamman et al., 2015). Significantly lower muscle protein synthesis (i.e., fractional synthetic rate) was observed in muscle surrounding the diseased hip in the MuIS$^{(+)}$ versus MuIS$^{(-)}$ groups (Bamman et al., 2015). The heightened inflammatory signaling and changes in muscle protein synthesis in the MuIS$^{(+)}$ versus MuIS$^{(-)}$ group would be expected to reduce myogenic activity and induce muscle atrophy. These findings suggest that dichotomization of individuals based on Fn14 expression reveals a phenotype that would be detrimental to recovery from THA, though long-term investigations evaluating the recovery potential of MuIS$^{(+)}$ versus MuIS$^{(-)}$ THA patients are currently lacking.

The possibility exists that these observations may not be unique to THA patients. Levinger et al. (2011) observed heightened inflammatory cytokine expression in the vastus lateralis of patients undergoing total knee arthroplasty (TKA) for end-stage osteoarthritis, suggesting the local inflammatory burden of an osteoarthritic knee joint similarly extends to surrounding musculature. Muscle inflammatory status may also predict muscle accretion and functional recovery potential following surgical repair of an acute orthopedic injury in otherwise healthy individuals. Anterior cruciate ligament (ACL) injury and reconstruction, for example, has been associated with elevated inflammatory cytokine expression in synovial fluid of the injured knee (Higuchi et al., 2006). Based on the findings in TKA patients, it is plausible that the local knee inflammation observed with ACL injury and reconstruction may similarly extend to the vastus lateralis. A heightened susceptibility to a locally inflamed joint (e.g., knee, hip, or shoulder) following musculoskeletal injury may contribute to an excessive inflammatory response to surgical treatment that limits regeneration of damaged tissue and promotes atrophy in the postoperative period. Whether this situation provides insight into a patient's rehabilitative potential is not known.

The MuIS$^{(+)}$ phenotype and age-related declines in regenerative capacity may have implications in the context of muscle injury given the associated muscle disuse atrophy and/or damage that demands a period of muscle growth for proper recovery. Short periods of immobilization or reduced physical activity are often necessary post-injury or postoperatively to

prevent further injury and facilitate recovery. While restricting motion allows the injured area to heal, muscle loss can occur rapidly under these conditions given the decline in loading and absence of neural activation (i.e., disuse atrophy). This is evident in experimental models of disuse that have shown a ∼4% loss of quadriceps cross-sectional area after only 5 days of unilateral knee immobilization in a population of young men (Wall et al., 2016), and a ∼4% loss of leg fat-free mass after 14 days of reduced daily step count (i.e., 6000 to 1500 steps per day) in a healthy older population (Breen et al., 2013). Rehabilitation begins gradually post-injury or post-operatively to attenuate muscle losses and regain muscle mass, strength, and function in the injured limb. While muscle hypertrophy during rehabilitation is primarily mediated by periods of positive muscle protein balance (i.e., muscle protein synthesis > muscle protein breakdown) that lead to lean mass accrual when maintained over time (Phillips, 2014), myogenesis likely plays a role.

While satellite cell involvement in muscle fiber regeneration has been consistently demonstrated, the role of satellite cells in muscle fiber hypertrophy and the effects of aging are less clear. Animal studies suggest that although muscle growth can occur in satellite cell ablated conditions (2 weeks overload) (McCarthy et al., 2011), satellite cells may be necessary to support more extensive muscle fiber hypertrophy (8 weeks overload) (Fry et al., 2014). Work in humans showing the upregulation of MRFs following resistance exercise (McKay et al., 2008; Nederveen et al., 2019), and positive correlations between muscle growth and increased satellite cell content during prolonged resistance exercise training (Petrella et al., 2006; Verdijk et al., 2010; Bellamy et al., 2014), has contributed to the idea that satellite cells play a role in human muscle fiber hypertrophy (Snijders et al., 2015; Brook et al., 2019). The MuIS$^{(+)}$ phenotype and age-related impairments in satellite cell function may therefore limit hypertrophy of skeletal muscle after an atrophy-inducing event. Suetta et al. (2013) showed that while a 4 week exercise intervention restored muscle mass lost due to 2 weeks of leg immobilization in young individuals (∼20 y), muscle fiber size was not recovered in an older population (∼70 y). Satellite cell expansion was also diminished during retraining in the old versus young individuals, suggesting an impaired regenerative response contributed to the lack of muscle restoration and repair (Suetta et al., 2013). Whether the MuIS$^{(+)}$ phenotype impairs muscle growth after injury-related muscle atrophy has not been determined.

Potential problems with existing methods of determining MuIS status that currently limit its clinical application should be noted. Dichotomization based on Fn14 expression, for example, results in groups of individuals (i.e., above versus below the cut-off point) that are considered equal although their individual prognosis may vary considerably. Likewise, individuals close to, but on opposite sides of, the cut-off point are regarded as very different. Dichotomization based on the sample median also means that the exact value of the cut-off point could change considerably from sample to sample. Using MuIS status effectively in clinical settings would require further exploration to determine a threshold of expression of specific markers (i.e., Fn14) that would be considered detrimental to muscle recovery potential. The clinical application of this tool in its current form also has methodical limitations. Collecting a muscle sample and evaluating gene expression is outside the scope of most clinical sites and laboratories. Finally, identifying appropriate therapeutic strategies to promote functional recovery and muscle mass accretion in individuals with the MuIS$^{(+)}$ phenotype would be necessary. Potential interventions may include more aggressive physical rehabilitation efforts (Bamman et al., 2015), or pharmacological treatment to attenuate excessive inflammation. Elucidating appropriate timing and targets for pharmacological interventions would be critical. Attenuating inflammation too early in the regeneration process may delay or diminish recovery. Likewise, targeting either pro-inflammatory or anti-inflammatory signaling, specifically, may disrupt the delicate balance of these pathways needed for proper regeneration. Treating chronic inflammation may instead require modulation of pathways involved in initiating the resolution of inflammation (Sugimoto et al., 2016). The potential benefits to determining MuIS status in orthopedic patient populations with specific regard for prognostic outcomes following surgical interventions warrants further investigation despite current limitations.

CONCLUSION

Skeletal muscle has the intrinsic capacity to regenerate and repair itself following injury through myogenesis, a process regulated by a finely controlled inflammatory response. This regenerative capacity is diminished with advanced age, however, as alterations in intrinsic and extrinsic signaling regulating satellite cell activity are observed in older adults. Cell and animal models also show that while inflammatory signaling mediates the myogenic response to muscle damage, high concentrations of several inflammatory cytokines (e.g., TNFα, IL-6, TWEAK) can inhibit muscle regeneration and trigger muscle wasting. Therefore advanced age and/or exceedingly high inflammatory signaling post-injury or postoperatively may limit the repair of damaged tissue and maintenance of muscle mass. Excessive or chronic inflammation is a particular concern for a subset of individuals who display elevated levels of basal intramuscular inflammatory signaling and greater susceptibility to an inflammatory insult (i.e., MuIS$^{(+)}$ phenotype). Future exploration of this interindividual variability in inflammatory susceptibility, focusing on understanding the cause of the observed differences, is warranted.

To date less is known about the divergent roles of inflammatory signaling following muscle injury in humans. Further investigations are needed to delineate the role of acute versus chronic inflammation as an anabolic or catabolic stimulus, respectively. Future work must also focus on molecular and cellular factors underlying the loss of regenerative capacity with aging. Understanding these processes is essential in developing clinical interventions to increase muscle regeneration and attenuate muscle atrophy after muscle injury in an effort to improve gains in muscle mass, strength, and function during rehabilitation. These interventions may also be relevant to the

treatment of chronic inflammatory myopathies characterized by diminished myogenesis and muscle wasting.

DISCLOSURE

The views and assertions expressed herein are those of the authors and do not reflect the official policy of the Army or the Department of Defense. Any citations of commercial organization and trade names in this report do not constitute an official Department of the Army endorsement of approval of the products or services of these organization.

AUTHOR CONTRIBUTIONS

EH, SP, and NR conceptualized the content of the article. EH wrote the original draft. EH, SP, CB, MF, and NR reviewed, edited, and approved the final version of the manuscript.

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Cancer Cachexia Induces Preferential Skeletal Muscle Myosin Loss When Combined With Denervation

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Patients with cancer cachexia (CCX) suffer from muscle wasting, which is often but not always accompanied by selective loss of myosin. Here we examined the effects of CCX on muscle mass and myosin heavy chain (MyHC) expression in denervated (DEN) muscles, especially focusing on the protein synthesis and degradation pathways. Male CD2F1 mice were randomly divided into control (CNT) and CCX groups and their left sciatic nerve was transected. CCX was induced by an intraperitoneal injection of colon 26 cells. After 14 days, the serum concentration of IL-6 and corticosteroid was higher in CCX mice than in CNT mice. The combination of CCX with DEN (CCX + DEN) resulted in a marked reduction of the gastrocnemius muscle weight (−69%) that was significantly lower than DEN (−53%) or CCX (−36%) alone. CCX had no effect on MyHC content, but it elicited a preferential MyHC loss when combined with DEN. The expression levels of autophagy markers cathepsin D and LC3BII/I ratio were markedly higher in the CCX + DEN group than in the CNT + DEN and the CCX groups. Paradoxically, there was an increase in protein synthesis rate and phosphorylation levels of p70S6K and rpS6, markers of mTORC1 signaling, in the CNT + DEN group, and these molecular alterations were inhibited in the CCX + DEN group. Our data indicate that CCX aggravates muscle atrophy in DEN muscles by inducing seletive loss of myosin, which involves inactivity dependent mechanisms that is likely to be a consequence of increased autophagy-mediated protein breakdown coupled with impaired protein synthesis.

Keywords: cancer cachexia, denervation, muscle atrophy, catabolism, anabolism

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INTRODUCTION

Cancer cachexia (CCX) is a multifactorial syndrome characterized by decreased skeletal muscle and adipose tissue mass (Tisdale, 2002). CCX is particularly profound in patients with gastrointestinal, lung, and pancreatic cancers, with about one-thirds of patients losing more than 5% of their baseline body weight (Dewys et al., 1980). This affects quality of life of the patients due to muscle weakness and fatigue and also has been associated with increased susceptibility to chemotherapy toxicity (Andreyev et al., 1998). Unlike starvation, in which adipose tissue is lost while lean body mass is preserved, nutritional supplementation fails to substantially reverse changes in body weight in CCX patients (Brennan, 1977; Evans et al., 1985). Accordingly, muscle wasting and cachexia have long been postulated as key determinants of cancer-related death (Zhou et al., 2010). Therefore,

understanding the cellular mechanisms behind the loss of muscle mass during CCX is highly significant from a clinical point of view.

It has been proposed that inflammatory cytokines are fundamental mediators of muscle atrophy in CCX. In cultured myotubes, inflammatory cytokines, including TNF-α, IL-1, and IL-6, directly bind surface receptors on muscle fiber driving muscle atrophy via the regulation of anabolic and catabolic pathways (Acharyya et al., 2004; Li et al., 2005, 2009; Bonetto et al., 2012). On the other hand, a major component of muscle atrophy in response to inflammation *in vivo* has been reported to be the results of the release of glucocorticoids that is likely to be induced by an activation of the hypothalamic-pituitary-adrenal axis (Braun et al., 2011). Strikingly, muscle-specific deletion of the glucocorticoid receptor affords a substantial protection against muscle atrophy associated with tumor growth (Braun et al., 2013).

Myosin is the most abundant protein and comprises about 30% of total muscle proteins. Each myosin molecule is composed of two myosin heavy chains (MyHCs) and four myosin light chains. Several studies have shown a preferential loss of MyHC in the animal models and the patients with CCX (Acharyya et al., 2004; Banduseela et al., 2007; Schmitt et al., 2007; Eley et al., 2008; Ochala and Larsson, 2008), suggesting a critical role of myosin loss in muscle wasting and weakness associated with CCX. Conversely, using the colon 26 (C-26) tumor bearing mice, we and others have demonstrated that all myofibrillar proteins decrease in parallel and that MyHC is not selectively reduced in cachectic muscles (Cosper and Leinwand, 2012; Tatebayashi et al., 2018). Thus, the impact of CCX on the regulation of MyHC expression is still under debate.

Intriguingly, denervation (DEN), and concomintant treatment of dexamethason (DEX), a synthetic glucocorticoid, produces characteristic pathologic feature of severe muscle atrophy, and preferential myosin depletion, although DEX treatment alone elicites muscle atrophy without loss of myosin (Rouleau et al., 1987; Mozaffar et al., 2007; Yamada et al., 2018, 2019). Given that CCX results in increased levels of glucocorticoid (Tanaka et al., 1990), these data imply that not only CCX but also inactivity would be necessary to induce selective loss of myosin, which may explain the conflicting findings regarding the levels of MyHC expression in CCX muscles. Furthermore, although muscle wasting is common end-points of CCX and inactivity, it has not been completely clarified whether these conditions are regulated by different mechanistic drivers. It is crucial therefore to deterimine whether the impacts of combined CCX and inactivity (e.g., DEN) are additive, which could have important clinical ramifications.

Muscle atrophy occurs when the overall rates of protein degradation exceed the rates of protein synthesis. Along with the time-course of muscle atrophy, a rapid loss of MyHC protein and mRNA as well as an activation of ubiquitin-proteasome system (UPS), a major protein degradation pathway, were shown to be induced within 3 days of DEN (Geiger et al., 2003; Sacheck et al., 2007). Despite a progressive decrease in muscle weight, a number of studies have reported a paradoxical increase in total RNA concentration, protein synthesis, and an activation of mTORC1 signaling, a primary translational regulator of muscle protein synthesis, following DEN (Mozaffar et al., 2007; Argadine et al., 2009; Quy et al., 2013). In contrast, DEX treatment does not inhibit MyHC mRNA induction (Mozaffar et al., 2007), but suppress the mTORC1 signaling (Schakman et al., 2013). Moreover, activation of autophagy pathway has been shown to be induced in DEX-treated mouse skeletal muscle (Penna et al., 2013). Hence, a better understanding of the anabolic and catabolic metabolism may provide mechanistic insights regarding the preferential myosin depletion seen in CCX condition.

C-26 tumor bearing mice are a commonly used animal model for CCX (Murphy et al., 2012). Intraperitoneal inoculation of C-26 tumor cells has been used as an animal model for peritoneal metastasis (Matsuyama et al., 2015), whereas C-26 tumor cells are generally inoculated subcutaneously into the flank of mice. Peritoneal metastasis develops in 8.5–25% of patients with colorectal cancer and is associated with severe muscle wasting (Jayne et al., 2002; Hilal et al., 2017). It has been demonstrated that tumor bearing mice inoculated into their intraperitoneal cavity shows a more severe CCX phenotype than those inoculated subcutaneously (Matsuyama et al., 2015). Thus, in the present study, we used the intraperitoneal C-26 tumor bearing mice and tested the following principle hypotheses: the impacts of combined CCX and DEN on muscle mass are additive; a preferential loss of myosin was induced by CCX in combination with DEN, but not CCX alone, and was due to a consequence of accererated protein breakdown concomitant with impaired protein synthesis.

MATERIALS AND METHODS

Ethical Approval

All experimental procedures were approved by the Committee on Animal Experiments of Sapporo Medical University (No. 18-110). Animal care was in accordance with institutional guidelines. A total of 13 mice (Sankyo Labo Service, Sapporo, Japan) were used in these experiments. At the end of the experiment, mice were killed by rapid cervical dislocation under anesthesia with 2% inhaled isoflurane to reach a stable anesthetic plane with consistent breathing rate and no response to toe pinch and muscles were subsequently isolated.

Experimental Design

Male CD2F1 mice (8 week old, $n = 13$) were supplied by Sankyo Labo Service (Sapporo, Japan) and were randomly assigned into control (CNT) ($n = 7$) and CCX ($n = 6$) groups. Mice were given food and water ad libitum and housed in an environmentally controlled room (24 ± 2°C) with a 12-h light-dark cycle. C-26 cells were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. The C-26 cells were cultured *in vitro* with RPMI-1640 supplemented with 10% (vol/vol) fetal bovine serum and 1% penicillin/streptomycin, and incubated at 37°C with 5% CO_2. CCX was induced by intraperitoneal injection of 5×10^6 C-26 cells diluted in 0.1 ml phosphate buffered saline and developed for 14 days, as described previously (Matsuyama et al., 2015). At the time of inoculation of

C-26 cells, DEN was induced on the left hindlimb (CNT + DEN and CCX + DEN) by removing a 10-mm segment of the sciatic nerve under 2% isoflurane anesthesia. Muscles from the right leg were used as innervated controls. After 14 days, blood samples were collected from the heart under isoflurane anesthesia and centrifuged at 3,000 rpm for 15 min, then serum was separated out and stored at −80°C for later analysis. Mice were killed by cervical dislocation under isoflurane anesthesia and the body weight, including intraperitoneal tumor, was measured. Then, the heart and the plantar flexor muscles were excised from each animal. Of note, it has been demonstrated that intraperitoneal inoculation of C-26 cells develops more rapid and severe CCX compared to subcutaneous inoculation of those cells and that the body weight of intraperitoneal tumor mice starts to decrease significantly around 1 week after inoculation (Matsuyama et al., 2015). Thus, these data suggest the concurrent presense of CCX and DEN at least 1 week before sacrifice in the CCX + DEN group in the present study.

ELISA

Corticosterone and interleukin (IL)-6 levels were measured in blood serum using corticosterone (No. 501320, Cayman Chemical) and IL-6 (KMC0061, ThermoFisher) ELISA kits according to manufacturer's instructions, respectively. A microplate reader was used to detect optical density of the colorimetric signal. The concentration of corticosterone and IL-6 was calculated by using recombinant mouse corticosterone and IL-6 as a standard, respectively.

Quantitative Real-Time PCR

Real-time PCR was used to quantify the mRNA levels for regulated in development and DNA damage responses (REDD) 1, forkhead box protein O3 (FoxO3), muscle-specific E3 ubiquitin ligases atrogin-1 and muscle ring finger 1 (MuRF-1) in frozen gastrocnemius (Gas) muscle tissue. Briefly, total RNA was extracted with TORIZOL reagent (Invitrogen, Carlsbad, CA), and the purity and yield of the total RNA extracted was determined by absorbance of aliquots at 260 and 280 nm (Thermo Scientific Nanodrop Light). Total RNA was reverse-transcribed to cDNA using Prime Script RT Reagent Kit (Takara, Japan). Synthesized cDNA was then amplified on the Applied Biosystems 7500 with Premix Ex Taq™ kit (Takara, Japan). The following Taqman Probes (Applied Biosciences™, Carlsbad, CA) were used: mouse REDD1 (Ddit4, Mm00512504_g1), mouse FoxO3 (Mm01185722_m1), mouse atrogin-1 (Fbxo32, Mm00499523_m1), mouse MuRF-1 (Trim63, Mm01185221_m1), mouse TATA (Tbp, Mm00446973_m1). All samples were run in duplicate. Relative amounts of target mRNA was determined using the comparative threshold cycle method (ΔΔCT). Expression of target genes was normalized to the corresponding expression level of TATA.

Measurement of Protein Synthesis

Muscle protein synthesis rate was measured in Gas muscles using a non-radioactive technique as described previously (Goodman et al., 2011). Briefly, puromycin (0.04 μmol/g body weight dissolved in 100 μl of PBS) was intraperitoneally injected into each animal, and then the Gas muscle was excised and quickly frozen in liquid nitrogen at exactly 30 min following the puromycin injection. The amount of puromycin incorporation into nascent peptide chains was determined by immunoblot analysis (see below).

Immunoblots

Immunoblots were performed using: anti-actin (A2172, Sigma Aldrich), anti-glutamine synthetase (GS) (GTX109121, GeneTex), anti-cathepsin D (ab75852, Abcam), anti-LC3B (ab63817, Abcam), anti-puromycin (MABE343, Merck Millpore), anti-total 70 kDa ribosomal S6 kinase (p70S6K) (9202, Cell Signaling), anti-p-p70S6K (Thr389) (9205, Cell Signaling), anti-total ribosomal protein S6 (rpS6) (2217, Cell Signaling), and anti-p-rpS6 (Ser240/244) (2215, Cell Signaling).

To extract whole muscle proteins, Gas muscle pieces were homogenized in ice-cold homogenizing buffer (30 μl/mg wet wt) consisting of (mM): Tris maleate, 10; NaF, 35; $NaVO_4$, 1; 1% Triton X 100 (vol/vol), and 1 tablet of protease inhibitor cocktail (Roche) per 50 ml. The protein content was determined using Bradford assay (Bradford, 1976). Aliquots of the whole muscle homogenates (15 μg) were diluted with SDS-sample buffer (mM): Tris/HCl, 62.5; 2% SDS (wt/vol); 10% glycerol (vol/vol); 5% 2-mercaptoethanol (vol/vol); 0.02% bromophenol blue (wt/vol). Proteins were separated on 4–15% Criterion TGX Stain Free gels (BioRad). Gels were imaged (BioRad Stain Free imager) and the ratio of MyHC to the total muscle proteins was measured by using Image Lab Software (BioRad). Then proteins were transferred onto polyvinylidine fluoride membranes. Membranes were blocked in 3% (wt/vol) non-fat milk, Tris-buffered saline containing 0.05% (vol/vol) Tween 20, followed by incubation with primary antibody overnight at 4°C. Membranes were then washed and incubated for 1 h at room temperature (∼24°C) with secondary antibody (1:10,000, donkey-anti-rabbit or donkey-anti-mouse, BioRad). Images of membrane were collected following exposure to chemiluminescence substrate (Millipore) using a charge-coupled device camera attached to ChemiDOC MP (BioRad), and Image Lab Software was used for detection as well as densitometry.

Statistics

Data are presented as mean ± SEM. Student's *t*-test was used to compare the body and heart weight and the concentration of corticosteroid and IL-6 between CNT and CCX mice. A two-way ANOVA, followed by the Tukey test for multiple comparisons, was used to determine statistically significant differences in muscle weight and the expression levels of mRNA and protein between the groups. A $P < 0.05$ was regarded as statistically significant. Statistical testing was performed with SigmaPlot (version 13, Systat Software, Inc.).

RESULTS

Cancer Cachexia Exacerbates Atrophy in Denervated Mouse Skeletal Muscles

Compared with CNT mice, the serum concentration of corticosteroid and IL-6 was increased in CCX mice [159 ± 4

vs. 236 ± 11 ng/ml (n = 5–7), $P < 0.05$; 1.35 ± 0.05 vs. 1.78 ± 0.14 pg/ml (n = 5–6), $P < 0.05$, respectively)]. Previous study has shown an increased serum IL-6 concentration as one of the cachexic phenotype, indicating the successful induction of CCX in C-26 tumor bearing mice (Matsuyama et al., 2015). Despite a distended abdomen, a sign of ascites, and extensive intraperitoneal tumor growth in CCX mice (**Figures 1A–C**), there was no difference in the body weight between the groups (**Figure 1D**), suggesting reduced tumor-free body mass in CCX mice. The heart weight of C-26 mice was significantly lower (−24%) than that of CNT mice ($P < 0.05$) (**Figure 1E**). Moreover, the weight for the fast-twitch Gas and plantaris muscles and slow-twitch soleus muscle was decreased by 36, 39, and 24% in the CCX group compared to the CNT group, respectively ($P < 0.05$) (**Figures 1F–H**). Similar results were obtained in DEN muscles; the weight for the Gas, plantaris, and soleus muscles was reduced by 46, 44, and 39% in the CNT + DEN group compared to the CNT group, respectively ($P < 0.05$). The weight for the Gas, plantaris, and soleus muscle in the CCX + DEN group was decreased by 69, 64, and 69% compared to the CNT group, respectively ($P < 0.05$), and was significantly lower than that of the CNT + DEN and the CCX groups ($P < 0.05$), indicating that the impacts of combined cachexia and inactivity on the regulation of muscle mass are additive.

Cancer Cachexia Induces Preferential Loss of Myosin in Denervated Mouse Gastrocnemius Muscles

Figure 2A shows a typical expression pattern of total muscle proteins and immunoblots for actin in the Gas muscles from each group. Compared to the CNT group, there were significant reductions in the MyHC content in the CNT + DEN (−37%) and the CCX + DEN (−60%) groups (**Figure 2B**). The degree of reductions in the MyHC content in the CCX + DEN group was much larger than that of the CNT + DEN group. The actin content in the CNT + DEN group was lower than that of the CNT group (**Figure 2C**). In contrast, there were no significant differences in MyHC and actin content between the CNT and the CCX group. Notably, the ratio of MyHC to actin was markedly lower in the CCX + DEN group than the CNT +DEN and the CCX groups (**Figure 2D**).

Cancer Cachexia Increases the Expression Levels of Ubiquitin Ligases mRNA and Autophagy-Related Proteins in Denervated Mouse Gastrocnemius Muscles

Expression levels of REDD1, FoxO3, MuRF-1, and atrogin-1 mRNA were markedly increased in Gas muscles from CCX mice compared with CNT mice (**Figures 3A–D**). Moreover, the expression levels of MuRF-1 and atrogin-1 mRNA in the CCX + DEN group were lower than those of the CCX group. The expression levels of GS and cathepsin D were increased in the CNT + DEN and CCX groups compared with the CNT group and were higher in the CCX + DEN group than those of the CNT + DEN and the CCX groups (**Figures 3E,F,H,I**). The LC3B-II/I ratio was markedly increasesd in the CCX + DEN group compared to the CNT + DEN and the CCX groups (**Figures 3G,J**).

Cancer Cachexia Inhibits the Activation of Protein Synthesis and the mTORC1 Signaling in Denervated Mouse Gastrocnemius Muscles

Compared to the CNT group, protein synthesis rate, as measured by puromycin incorporation into protein, was increased in the DEN group, while it was reduced in the CCX group (**Figures 4A,B**). The protein synthesis rate was higher in the CCX + DEN group than in the CCX group. The phosphorylation levels of p70S6K Thr389 and rpS6 Ser240/244 were markedly increased in the DEN group compared to the CNT group (**Figures 4A,C,D**). Interestingly, both protein synthesis rate and the phosphorylation levels of p70S6K and rpS6 was lower in the CCX + DEN group than in the DEN group.

DISCUSSION

To our knowledge, this is the first study directly showing that CCX accelerates muscle atrophy in DEN muscles. Moreover, a preferential myosin loss was induced by CCX in combination with inactivity, but not CCX alone, in rodent skeletal muscles. These findings indicate that CCX and inactivity are likely to be regulated by different mechanistic drivers and that CCX-induced selective loss of myosin involves inactivity dependent mechanisms.

The degree of Gas muscle atrophy in the intraperitoneal tumor mice in the present study (−36%) was much greater than that of the subcutaneous tumor mice in our previous study (−11%) (Tatebayashi et al., 2018). These results are consistent with the study by Matsuyama et al. (2015), which showed that the weight loss in Gas muscle is more marked in the intraperitoneal tumor mice than in the subcutaneous tumor mice. Moreover, in line with their findings, myocardium weight loss and increased circulating cytokines were observed in the intraperitoneal tumor mice, suggesting the severe systemic inflammation in those mice. These data suggest that intraperitoneal tumor mice, an animal model for peritoneal metastasis, would be an improved model to study CCX-induced muscle wasting compared to the established subcutaneous tumor mice.

Glucocorticoid appears to be a major determinant of inflammation-induced muscle atrophy and to play an important role in the pathogenesis of CCX (Schakman et al., 2008; Braun et al., 2013). In agreement with the previous studies, we found a marked increase in serum IL-6 (Bonetto et al., 2012) and corticosterone (Tanaka et al., 1990) concentration in CCX mice. FoxO3 has been proposed to regulate autophagy in glucocorticoid-treated and C-26 tumor bearing mice (Penna et al., 2013). Moreover, van der Vos et al. (2012) revealed GS as a FoxO3 target gene that regulate autophagy. It has been demonstrated that a combination of DEN and DEX resulted in additive increases in GS activity (Feng et al., 1990), which is consisitent with our present findings. The mechanism underlying increased GS after DEN appears to be posttranscriptional and is

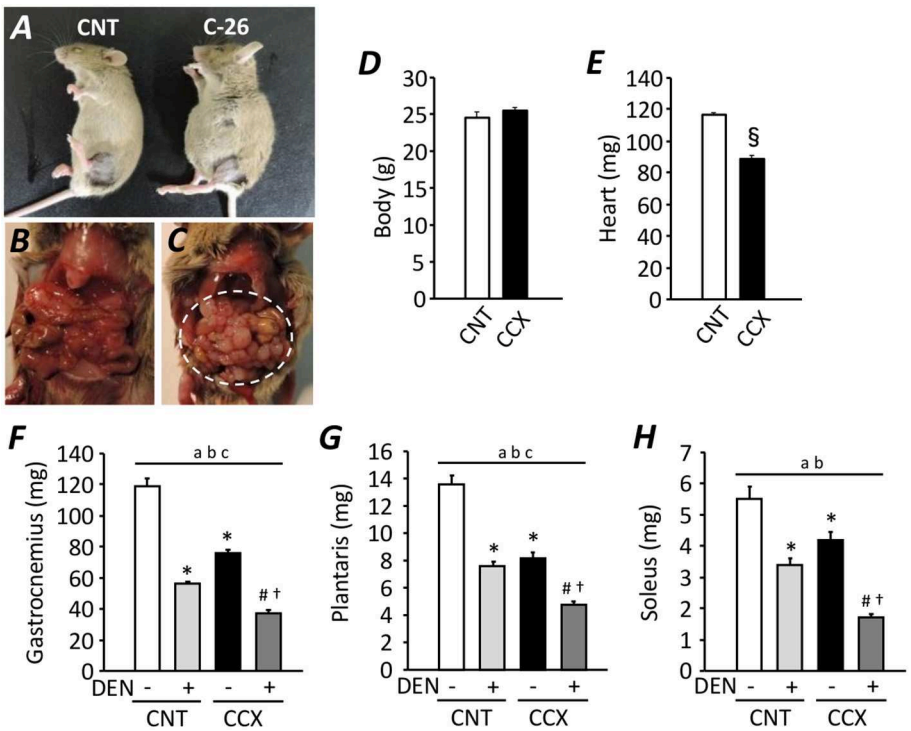

FIGURE 1 | Cancer cachexia exacerbates atrophy in denervated mouse skeletal muscles. Photographs of anesthetized control (CNT) and cancer cachexia (CCX) mice **(A)**. Images after mouse laparotomy in CNT **(B)** and CCX mice **(C)**. Note that CCX mice showed extensive intraperitoneal tumor growth (dotted circle). The body **(D)** and heart **(E)** weight in each group. Bars show the mean and SEM results from 6 to 7 mice per group. $^{\S}P < 0.05$ vs. CNT. The muscle wet weight of fast-twitch gastrocnemius **(F)** and plantaris muscles **(G)** and slow-twitch soleus muscles **(H)** from CNT and CCX mice, with or without denervation (DEN). Bars show the mean and SEM results from 6 to 7 muscles per group. Statistical significance was set at $P < 0.05$: main effect of [a]CCX and [b]DEN; [c]interaction of CCX and DEN; difference vs. *CNT without DEN, #CNT with DEN, and †CCX without DEN.

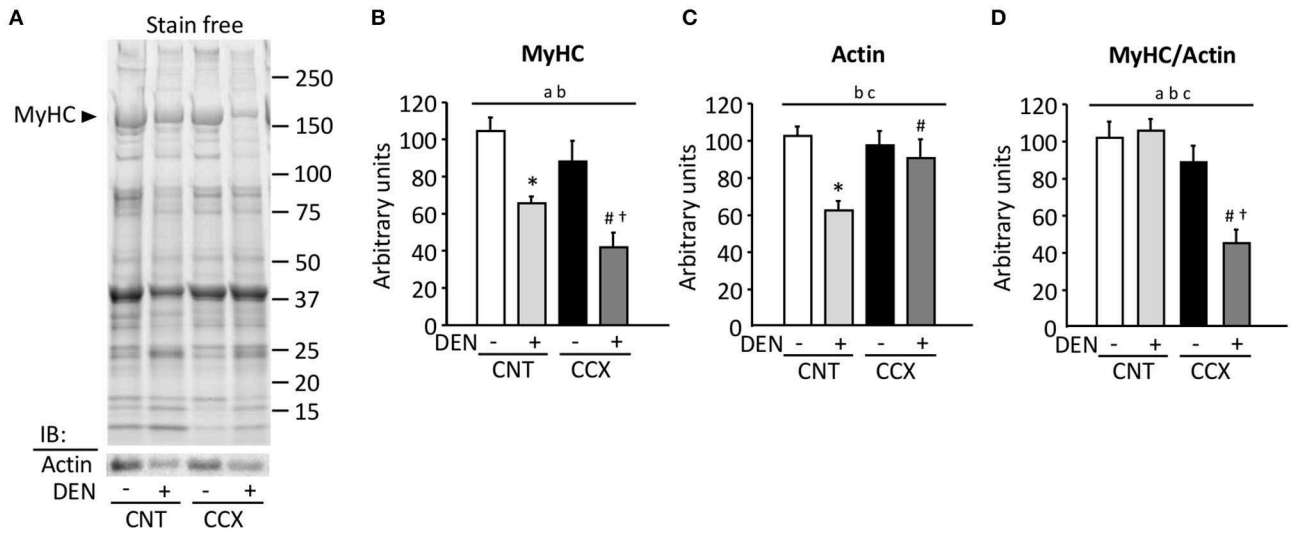

FIGURE 2 | Cancer cachexia induces preferential loss of myosin in denervated mouse gastrocnemius muscles. Representative stain free images of whole muscle proteins and immunoblots for actin in gastrocnemius muscles from control (CNT) and cancer cachexia (CCX) mice, with or without denervation (DEN) **(A)**. Myosin heavy chain (MyHC) **(B)** and actin **(C)** content was normalized by total muscle proteins. The ratio of MyHC to actin **(D)**. Bars show the mean and SEM results from 5 to 6 muscles per group. Statistical significance was set at $P < 0.05$: main effect of [a]CCX and [b]DEN; [c]interaction of CCX and DEN; difference vs. *CNT without DEN, #CNT with DEN, and †CCX without DEN.

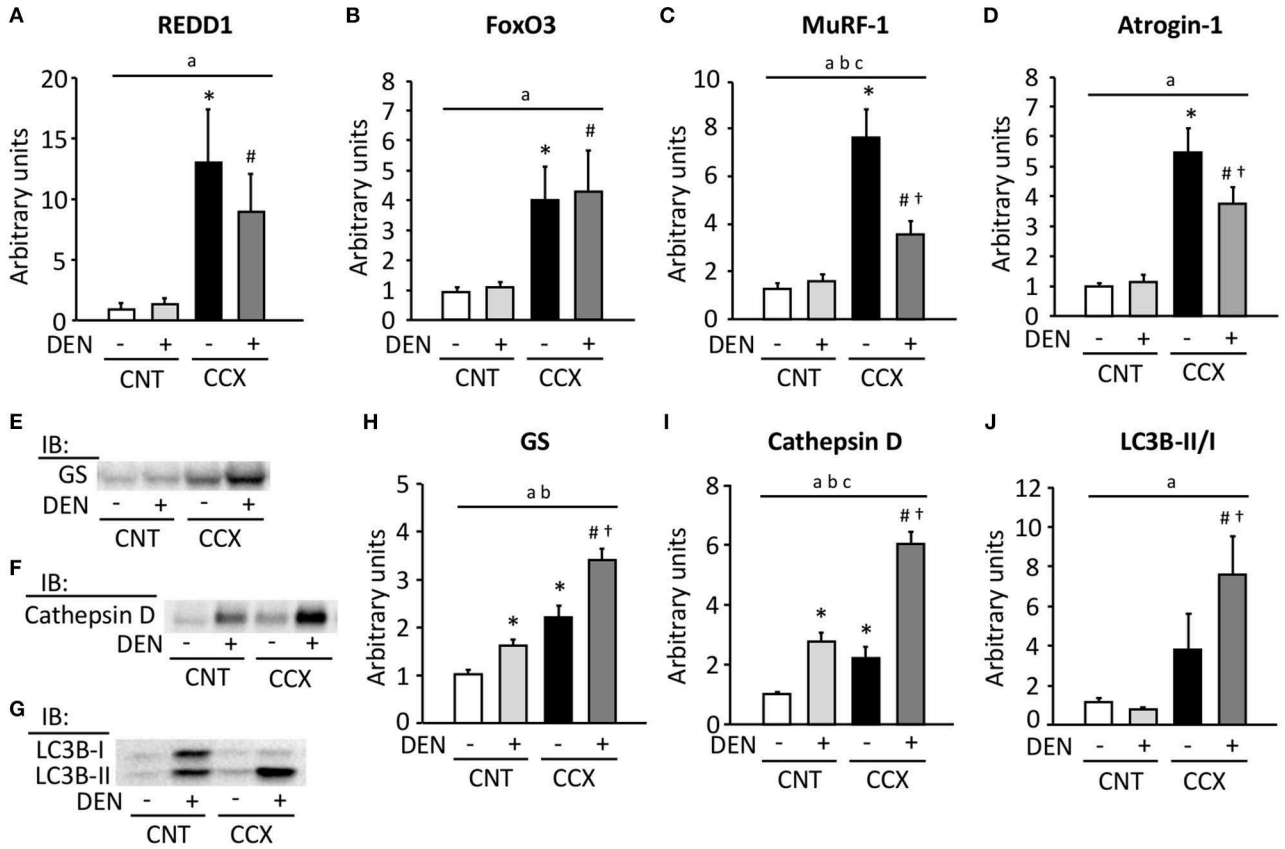

FIGURE 3 | Cancer cachexia increases the expression levels of ubiquitin ligases mRNA and autophagy-related proteins in denervated mouse gastrocnemius muscles. The expression levels of regulated in development and DNA damage responses (REDD) 1 **(A)**, forkhead box O (FoxO) 3 **(B)**, muscle ring finger protein 1 (MuRF-1) **(C)**, and atrogin-1 **(D)** mRNA in gastrocnemius muscles from control (CNT) and cancer cachexia (CCX) mice, with or without denervation (DEN), were normalized to the TATA-binding protein mRNA and expressed as fold change of the mean CNT value, which was set to 1. Representative western blots for glutamine synthetase (GS) **(E)**, cathepsin D **(F)**, and microtube-associated protein 1 light chain 3B (LC3B)-I and -II **(G)**. The expression levels of GS **(H)** and cathepsin D **(I)** were normalized to the total protein content seen in the stain free images. The LC3B-II/LC3B-I ratio **(J)**. Data show mean ± SEM for 5–7 muscles per group. Statistical significance was set at $P < 0.05$: main effect of [a]CCX and [b]DEN; [c]interaction of CCX and DEN; difference vs. *CNT without DEN, #CNT with DEN, and †CCX without DEN.

distinct from that of the glucocorticoid-mediated transcriptional regulation of GS induction (Feng et al., 1990). Thus, these data suggest that an activation of glucocorticoid/FoxO3/autophagy network appears to be involved in CCX-induced muscle atrophy.

Paradoxically, protein synthesis, and mTORC1 pathway were activated in DEN muscles, in agreement with what previously reported by others (Argadine et al., 2011; Machida et al., 2012; Quy et al., 2013). It has been suggested that DEN-induced mTORC1 activation is dependent on the proteasome, which is considered to be mediated by amino acids generated from proteasomal degradation (Quy et al., 2013). However, in our study where muscles were denervated for 14 days, activation of mTORC1 signaling was not accompanied by increased mRNA levels of E3 ubiquitin ligases, implying limited intracellular concentration of amino acid at this time point. Thus, although an activation of anabolic signaling raises the possibility that muscle disuse induces compensatory mechanisms to maintain muscle mass and limit atrophy in the absence of innervation (Wang et al., 2005), this may not depend on induction of amino acid by proteasomal degradation. Conversely, CCX suppressed protein synthesis rate, as measured by puromyocin incorporation into protein, which is consistent with our previous study (Tatebayashi et al., 2018). In addition, CCX inhibited increased protein synthesis rate induced by DEN, suggesting that CCX may disrupt compensatory adaptation (i.e., activation of anabolic pathway) in DEN muscles. Thus, although the reduced protein synthesis rate in CCX muscles was restored by DEN treatment, this may not be enough to counteract protein catabolism and hence muscle atrophy was accelerated in DEN muscles from CCX mice.

It is widely accepted that mTORC1 is a major suppressor of autophagy (Kroemer et al., 2010; Mizushima, 2010). Coffer and colleagues (van der Vos et al., 2012) demonstrated that increased levels of GS inhibit mTOR signaling by its lysosomal translocation, which lead to activation of autophagosome formation. Consistent with this, the remarkable increase in GS expression was accompanied by inhibition of DEN-induced hyperphosphorylation of p70S6K and augmentation of LC3BII/I ratio, a marker of autophagosome production, in the CCX + DEN muscles. Additionally, upregulation of cathepsin D, a protease associated with the lysosome autophagy system, implies

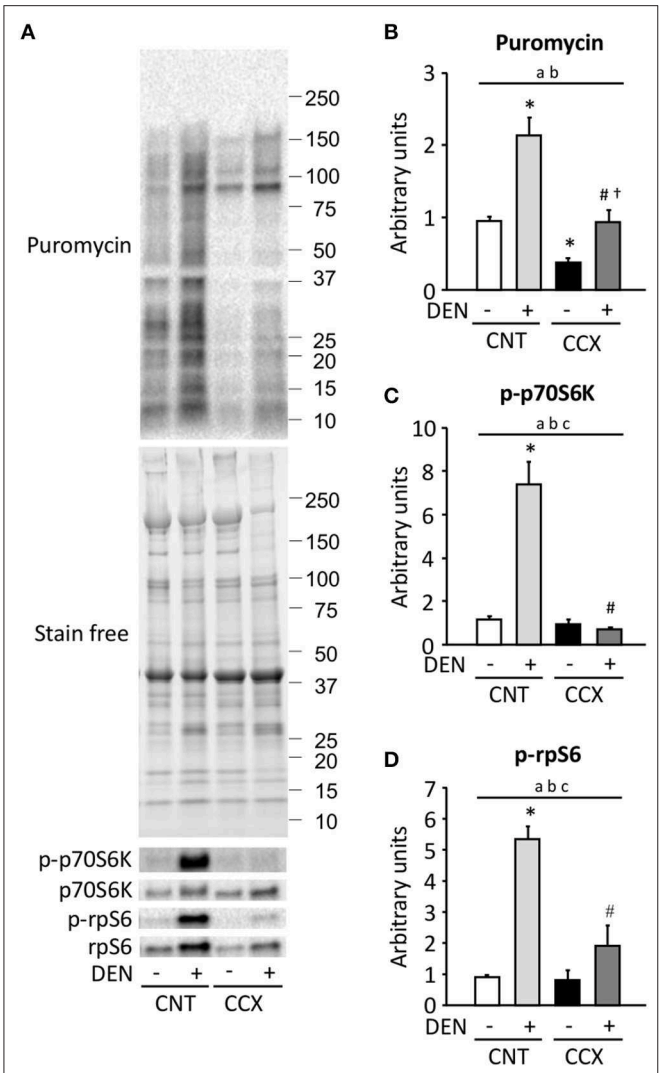

FIGURE 4 | Cancer cachexia inhibits the activation of protein synthesis and the mTORC1 signaling in denervated mouse gastrocnemius muscles. Representative western blots for puromycin, total and phosphorylated p70S6K Thr389 (p-p70S6K), and total and phosphorylated rpS6 Ser240/244 (p-rpS6) in control (CNT) and cancer cachexia (CCX) mice, with or without denervation (DEN) **(A)**. The expression levels of puromycin was normalized to the whole proteins in stain free images and expressed as fold change of the mean CNT value, which was set to 1 **(B)**. The levels of p-p70S6K **(C)** and p-rpS6 **(D)** were normalized to total p70S6K and rpS6 content, respectively. Data show mean ± SEM for 6–7 muscles per group. Statistical significance was set at $P < 0.05$: main effect of [a]CCX and [b]DEN; [c]interaction of CCX and DEN; difference vs. *CNT without DEN, #CNT with DEN, and †CCX without DEN.

an increase in autophagosome clearance in the CCX + DEN group. Importantly, previous study has revealed that cathepsin D selectively degrades MyHC in the purified myofibrils from rabbit muscles (Okitani et al., 1981). Thus, these data suggest that CCX aggravates muscle atrophy and preferential myosin loss by activation of autophagy flux in DEN muscles.

Our findings showed that markers of UPS are enhanced in the CCX group in comparison to the CNT + DEN group, while those of autophagy are comparable between these groups. Despite this difference, however, the degree of muscle atrophy was similar between CCX and DEN muscles. This discrepancy may be partly related to the difference in the time-course change in UPS response between DEN and CCX muscles. It has been demonstrated in rat DEN muscles that the two E3 ubiquitin ligases, MuRF1 and atrogin-1, are markedly upregulated at 3 days, when rates of muscle weight loss are the highest, and are returned toward basal levels at 14 days after DEN as the rate of atrophy slowed (Sacheck et al., 2007). These data can explain our findings that neither MuRF1 nor atrogin-1 mRNA were upregulated even though significant wasting was seen in 14 days after DEN. On the other hand, MuRF1 and atrogin-1 mRNA were increased in CCX muscles 14 days after tumor inoculation presumably due to the cachexic phenotype which developed with time in C-26 tumor bearing mice. Suprisingly, despite the upregulation of E3 ubiquitin ligases, CCX alone did not elicite a reduction in myosin content. Although it is well-known that degradation of myofibrillar proteins, such as MyHC, is mainly mediated by the UPS (Sandri, 2008), MuRF1 is regarded as a general ligase affecting many contractile, soluble, and nuclear proteins (Cohen et al., 2009). Therefore, the selective loss of myosin observed in the CCX + DEN muscles is unlikely to be due to the activation of UPS.

Notably, DEN treatment partially suppressed the increased mRNA levels of MuRF-1 and atrogin-1 induced by CCX. Although the reason for these molecular adaptations is unclear, it can be interpreted as an inhibitory effect of DEN on proteasome-mediated muscle protein breakdown in CCX muscles. However, muscle mass was markedly reduced in the CCX + DEN group compared with the CCX group, suggesting that the activation of UPS is not the predominant cause and other pathways are likely to be responsible for the striking decrease in muscle mass in such a situation. In this regard, Wang et al. (2005) have demonstrated that Runx1 (AML1), a DNA-binding protein, is strongly induced in DEN muscles and is required to sustain muscle mass by preventing DEN muscles from autophagy. Interestingly, they suggested that CCX may cause a failure in DEN-induced upregulation of Runx1, resulting in severe muscle wasting accompanied by hallmarks of autophagy. The autophagic-lysosomal pathway has been suggested to be the main proteolytic system in skeletal muscle from non-active cachectic patients with esophageal cancer (Tardif et al., 2013). Thus, these findings further highlight an importance of autophagy pathway in DEN muscles from CCX mice. However, our conclusions regarding mechanism are based on correlations and further studies with pharmacological or genetic inhibition of autophagy at the time of C-26 inoculation and/or DEN would more directly clarify the molecular mechanisms of muscle wasting in the DEN muscles from CCX mice.

Interestingly, DEX treatment has been shown to result in a preferential loss of MyHC in denervated rat skeletal muscle (Rouleau et al., 1987; Mozaffar et al., 2007). A similar picture emerged in our study where selective loss of MyHC was induced by CCX when combined with DEN. Previous study has shown that DEN treatment reduces the expression levels of MyHC and actin mRNA in rat skeletal muscle (Mozaffar et al.,

2007). In contrast, our data demonstrated that CCX upregulates REDD1 mRNA and suppresses protein synthesis and mTORC1 pathway in DEN muscles. REDD1 is known to be induced by glucocorticoid and to inhibit anabolic signaling (Britto et al., 2014). Therefore, DEN-induced transcriptional defects coupled with glucocorticoid-induced translational inhibition may be attributed to the preferential myosin depletion in DEN muscles from CCX mice.

CONCLUSIONS

The present results clearly show that the impacts of combined CCX and DEN (i.e., inactivity) on the regulation of muscle mass are additive. Moreover, a preferential myosin loss is induced by CCX when coupled with DEN, but not by CCX alone. Our findings suggest that CCX may disrupt inactivity-induced compensatory mechanisms that preserve muscle mass and limit fiber atrophy, resulting in severe muscle wasting, and selective myosin loss due to increased autophagy-mediated protein breakdown concomitant with impaired protein synthesis.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by the Committee on Animal Experiments of Sapporo Medical University (No. 18-110).

AUTHOR CONTRIBUTIONS

TY contributed to the conception and design of the study. TY, YA, DT, MA, and KH participated in the analysis and interpretation of the data. TY, YA, and DT were responsible for data collection. TY was involved in writing the manuscript and all authors approved the final version. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed. All experiments were performed at the Muscle Physiology Laboratory in the Graduate School of Health Sciences, Sapporo Medical University, Sapporo, Japan.

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The Effects of Cold Water Immersion and Active Recovery on Molecular Factors That Regulate Growth and Remodeling of Skeletal Muscle After Resistance Exercise

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Regular postexercise cooling attenuates muscle hypertrophy, yet its effects on the key molecular factors that regulate muscle growth and remodeling are not well characterized. In the present study, nine men completed two sessions of single-leg resistance exercise on separate days. On 1 day, they sat in cold water (10°C) up to their waist for 10 min after exercise. On the other day, they exercised at a low intensity for 10 min after exercise. Muscle biopsies were collected from the exercised leg before, 2, 24, and 48 h after exercise in both trials. These muscle samples were analyzed to evaluate changes in genes and proteins involved in muscle growth and remodeling. Muscle-specific RING finger 1 mRNA increased at 2 h after both trials ($P < 0.05$), while insulin-like growth factor (IGF)-1 Ec, IGF-1 receptor, growth arrest and DNA damage-inducible protein 45, collagen type I alpha chain A, collagen type III alpha chain 1, laminin and tissue inhibitor of metallopeptidase 1 mRNA increased 24−48 h after both trials ($P < 0.05$). By contrast, atrogin-1 mRNA decreased at all time points after both trials ($P < 0.05$). Protein expression of tenascin C increased 2 h after the active recovery trial ($P < 0.05$), whereas FoxO3a protein expression decreased after both trials ($P < 0.05$). Myostatin mRNA and ubiquitin protein expression did not change after either trial. These responses were not significantly different between the trials. The present findings suggest that regular cold water immersion attenuates muscle hypertrophy independently of changes in factors that regulate myogenesis, proteolysis and extracellular matrix remodeling in muscle after exercise.

Keywords: exercise, recovery, cryotherapy, extracellular matrix, adaptation, atrogenes

INTRODUCTION

Athletes commonly use cold water immersion to recover after intense exercise, based on the belief that it provides physiological benefits that expedite return to training and competition. However, mounting evidence indicates that when used regularly, cold water exercise can diminish long-term gains in strength and muscle mass after strength training (Peake, 2020). Research into the mechanisms responsible for this effect has revealed that acute cold water immersion after resistance reduces or interferes with several important acute processes and pathways that stimulate muscle hypertrophy, including: muscle protein synthesis, the expression of genes that regulate intracellular amino acid transport, satellite cell proliferation, phosphorylation of kinases in the mTOR and p38-MNK1-eIF4E signaling pathways, and ribosomal DNA transcription (Roberts et al., 2015; Figueiredo et al., 2016; Fyfe et al., 2019; Fuchs et al., 2020). Regular cold water immersion may also attenuate chronic changes in heat shock proteins, while also activating factors responsible for catabolism in muscle [e.g., Forkhead box O (FoxO)] (Fyfe et al., 2019).

Cold water immersion could also influence long-term gains in strength and muscle mass through other mechanisms. In addition to the mTOR and p38-MNK1-e1F4E pathways, muscle growth is regulated through the IGF-1−PI3K−Akt pathway (Schiaffino et al., 2013). The activity of these growth-related pathways in skeletal muscle is balanced by the myostatin−Smad3 and ubiquitin−proteasome pathways, which promote muscle proteolysis (Schiaffino et al., 2013). Resistance exercise activates the IGF-1−PI3K−Akt, myostatin−Smad3 and ubiquitin−proteasome pathways. The gene expression of the IGF-1 isoforms IGF-1Ea, IGF-1Eb, and IGF-1Ec (also known as mechano growth factor) and the IGF-1 receptor increases acutely in skeletal muscle after resistance exercise (Psilander et al., 2003; McKay et al., 2008; Wilborn et al., 2009; Heinemeier et al., 2013). Among the negative regulators of muscle growth, muscle-specific RING finger (MuRF)-1 mRNA increases acutely (Louis et al., 2007; Drummond et al., 2008; Mascher et al., 2008; Reitelseder et al., 2014; Stefanetti et al., 2014; Zak et al., 2018), as does the expression of genes encoding various proteins in the ubiquitin−proteasome pathway (Mahoney et al., 2008). By contrast, myostatin expression decreases (Louis et al., 2007; Deldicque et al., 2008; Drummond et al., 2008; Mascher et al., 2008; Wilborn et al., 2009; Zak et al., 2018). Acute changes in the expression of atrogin-1 (also known as muscle atrophy F-box or F-box protein 32) (Louis et al., 2007; Deldicque et al., 2008; Mascher et al., 2008; Manini et al., 2011; Nader et al., 2014; Reitelseder et al., 2014; Stefanetti et al., 2014; Zak et al., 2018) and FoxO (Louis et al., 2007; Manini et al., 2011; Reitelseder et al., 2014; Stefanetti et al., 2014; Zak et al., 2018; Fyfe et al., 2019; Fuchs et al., 2020) in skeletal muscle after resistance exercise are more variable and time-dependent. The gene expression of growth arrest and DNA damage-inducible protein (GADD45) also increases acutely in skeletal muscle after eccentric exercise (Chen et al., 2003; Mahoney et al., 2008) and endurance exercise (Romero et al., 2016). GADD45 plays a role in both muscle growth (Carson et al., 2002) and atrophy (Ebert et al., 2012).

Together with alterations in these growth factors and so-called "atrogenes" (that mainly regulate protein content in muscle fibers), exercise also induces remodeling of the extracellular matrix (ECM) in skeletal muscle. Exercise stimulates mRNA expression of collagen type I alpha 1 chain, collagen type III alpha 1 chain, matrix metalloproteinases, tissue inhibitor of metallopeptidase, tenascin C mRNA and protein in skeletal muscle (Crameri et al., 2004, 2007; Mikkelsen et al., 2011; Hoier et al., 2012; Heinemeier et al., 2013; Hyldahl et al., 2015). As a result of these changes, collagen formation increases in skeletal muscle after exercise (Crameri et al., 2004; Miller et al., 2005) supporting the repair and growth of new myofibers (Mackey and Kjaer, 2017).

Two animal studies have reported that icing attenuates (Vieira Ramos et al., 2016) or delays (Takagi et al., 2011) the expression of genes or proteins involved in muscle remodeling after injury. Two recent human studies have demonstrated that cold water immersion reduced the expression of phosphorylated $FoxO1^{Ser256}$ (but not the expression of phosphorylated $FoxO3a^{Ser253}$) (Fyfe et al., 2019) whereas it did not alter mRNA expression of FOXO1, MuRF1 or atrogin-1 in muscle after resistance exercise (Fuchs et al., 2020). Some gaps and inconsistencies therefore exist in the literature about how cryotherapies such as icing or cold water immersion influence factors involved in muscle remodeling after exercise and/or muscle injury.

In the present study, we compared how acute cold water immersion and active recovery influence the expression of growth factors, ubiquitin−ligases, myostatin, FoxO and ECM genes and proteins in skeletal muscle after resistance exercise. To achieve this objective, we analyzed muscle samples that we collected as part of a larger study (Roberts et al., 2015). We hypothesized that cold water immersion would attenuate the expression of growth factors and ECM genes/proteins, and enhance the expression of ubiquitin ligases and FoxO3a.

MATERIALS AND METHODS

Ethical Approval

All participants were informed of the requirements and potential risks of the study before providing their written informed consent. The experimental procedures met the standards described in the Declaration of Helsinki, and were approved by the Human Research Ethics Committee of the University of Queensland.

Experimental Design

We have reported details and the characteristics of the participants of this study elsewhere (Roberts et al., 2015). Nine young, healthy and physically active men (mean ± SD age 22.1 ± 2.2 years, height 1.80 ± 0.06 m, body mass 83.9 ± 15.9 kg) volunteered to participate in the study. The inclusion criteria for the study were that participants were healthy, non-smokers with at least 12 months of experience in strength training, and were not taking any regular medication (e.g., analgesic or anti-inflammatory drugs) that could influence signaling

pathways in skeletal muscle. The experimental design comprised a randomized, cross-over study in which the participants performed bouts of single-leg resistance exercise on two separate occasions. After one session, the participants immersed their lower body in cold water for 10 min, while after the other session they exercised at a low intensity for 10 min. The order of these two trials was randomized and counterbalanced among the participants. Muscle biopsies were collected from *vastus lateralis* of the exercised leg before, 2, 24, and 48 h after each exercise session.

Strength Exercises

The exercise protocol for the two experimental trial was the same, and involved a combination of lower-body exercises (e.g., 45° leg press, single leg squats, knee extensions and walking lunges). These exercises were performed at loads of 8, 10, and 12 repetition maximum. All strength training was supervised, and was performed in a gymnasium in which the ambient temperature was 23–25°C.

Recovery Therapies

In the cold water immersion trial, the participants sat in an inflatable bath (iCool iBody, iCool, Miami, Australia) for 10 min with both legs immersed in water up to the waist. Water was circulated continuously and was maintained at 10.3 ± 0.5°C using a circulatory cooling unit (iCool LITE, iCool). In the active recovery trial, the participants exercised for 10 min active recovery at a self-selected low intensity (36.6 ± 13.8 W) on a stationary cycle ergometer (Wattbike, Nottingham, United Kingdom). To restrict any re-warming following cold water immersion or cooling following active recovery, the participants were prevented from showering or bathing for at least 2 h after the recovery therapies.

Muscle Tissue Collection

Muscle biopsies were collected from the mid portion of the vastus lateralis of one leg before exercise and again at 2, 24 and 48 h after each exercise trial. Pre-exercise and 2 h post-exercise biopsies were obtained from the same site on the leg. For the pre-exercise biopsy, the biopsy needle was inserted in a distal direction, whereas for the 2 h biopsy, the needle was inserted in a proximal direction. This method ensured a distance of at least 3 cm between the two biopsy sites. Biopsies at 24 and 48 h were collected from separate sites, each ∼3 cm proximal from the previous site. After the muscle biopsies were taken, the muscle tissue was rinsed with 0.9% saline. Any fat, connective tissue or blood was removed before the sample was frozen in isopentane cooled in dry ice. Samples were stored at −80°C until analysis.

Control Procedures

We restricted possible variation in training responses by providing standardized nutrition after each training session, and asking the participants not to exercise for 72 h prior to each trial. Before each trial, the participants ate the same meal 2 h before the pre-exercise muscle biopsy. Before each recovery treatment, they were given a drink containing 30 g serve of whey protein isolate. After consuming this drink, they were only permitted to drink water until the 2 h biopsy was collected. At that time, they were given another 30 g of whey protein isolate to drink. The participants were asked not to take supplements of any kind between 4 d before each pre-exercise biopsy, and the 48 h post-exercise muscle biopsy. The participants were encouraged to adhere to their regular diet for 2 days before each experimental trial and until the 48 h muscle biopsy. Dietary intake before and during the first experimental trial was recorded in a food diary and replicated for the second experimental trial. The participants did not exercise until after the 48 h muscle biopsy for each trial.

Muscle Tissue Analysis

RT-PCR

Total RNA was extracted from ∼20 mg of muscle tissue using the AllPrep® DNA/RNA/miRNA Universal Kit (QIAGEN GmbH, Hilden, Germany). cDNA was prepared using High-Capacity RNA-to-cDNA™ kit (Life Technologies, Carlsbad, CA, United States). Messenger RNA (mRNA) was quantified using RT-PCR on a LightCycler 480 II (Roche Applied Science, Penzberg, Germany) using SYBR Green I Master Mix (Roche Applied Science). **Table 1** describes the sequences for the primers used in this study. To normalize the RT-PCR data, the geometric mean of three housekeeping genes was used (Vandesompele et al., 2002). Standard and melting curves were performed for each target to determine primer efficiency and single product amplification.

Western Blotting

To measure FoxO3a protein expression, portions of muscle tissue (45−55 mg) were homogenized and separated into cytosolic and nuclear fractions using a commercial kit (ProteoExtract Subcellular Proteome Extraction Kit, Calbiochem, EMD Biosciences, Germany). The purity of the fractions was evaluated using specific markers for the respective fractions [i.e., GAPDH (cytosol and nuclear) and PARP (nuclear)]. Protein concentration in the samples was quantified in triplicate using a kit (DC Protein Microplate assay, Bio-Rad, Hercules, CA, United States), a filter photometer (Expert 96, ASYS Hitech, United Kingdom), and the software provided (Kim, ver. 5.45.0.1, Daniel Kittrich, Prague, Czechia).

Equal amounts of protein were loaded into each well (11 μg for the nuclear fraction and 20 μg for the cytosolic fraction) and were separated by 4−12% SDS-PAGE under denaturizing conditions for 35−45 min at 200 V in cold MES running buffer (NuPAGE MES SDS Running Buffer, Invitrogen, Carlsbad, CA, United States). All samples were analyzed in duplicate. Following gel electrophoresis, the proteins were transferred onto a polyvinylidene fluoride membrane for 90 min at 30 V using an XCell II Blot Module (Thermo Fisher Scientific, Hemel Hempstead, United Kingdom) and NuPAGE transfer buffer (Invitrogen, Carlsbad, CA, United States). Membranes were blocked at room temperature for 2 h in a 5% fat-free skimmed milk and 0.1% Tris–buffered saline with Tween 20 (TBST) (Bio-Rad; Tween-20, VWR International, Radnor, PA, United States; skim milk, Merck, Darmstadt, Germany). Blocked membranes were incubated overnight at 4°C with a primary monoclonal antibody against FoxO3a (Cell Signaling Technology, Danvers, MA, United States), diluted 1:400. After incubation, membranes

TABLE 1 | mRNA primer sequences.

Primer	Sequence
IGF1R Forward	CTCCTGTTTCTCTCCGCCG
IGF1R Reverse	ATAGTCGTTGCGGATGTCGAT
IGF1Ec (MGF) Forward	CGAAGTCTCAGAGAAGGAAAGG
IGF1Ec (MGF) Reverse	ACAGGTAACTCGTGCAGAGC
IGF1Ea Forward	GACATGCCCAAGACCCAGAAGGA
IGF1Ea Reverse	CGGTGGCATGTCACTCTTCACTC
GADD45A Forward	CGATAACGTGGTGTTGTGCC
GADD45A Reverse	GTTGATGTCGTTCTCGCAGC
GADD45B Forward	GGGAAGGTTTTGGGCTCTCT
GADD45B Reverse	GGTCACCGTCTGCATCTTCTG
Atrogin1 Forward	AATAAGGAGAATCTTTTCAACAGCC
Atrogin1 Reverse	TCCATGGCGCTCTTTAGTACTTC
MuRF1 Forward	GGGACAAAAGACTGAACTGAATAAC
MuRF1 Reverse	GGCTCAGCTCTTCCTTTACCT
Myogenin Forward	GGCCAAACTTTTGCAGTGAATATT
Myogenin Reverse	TCGGATGGCAGCTTTACAAACAAC
Myostatin Forward	CTACAACGGAAACAATCATTACCA
Myostatin Reverse	GTTTCAGAGATCGGATTCCAGTAT
Collagen type I alpha chain 1 Forward	TGAAGGGACACAGAGGTTTCAG
Collagen type I alpha chain 1 Reverse	GTAGCACCATCATTTCCACGA
Collagen type III alpha chain 1 Forward	GAAAGATGGCCCAAGGGGTC
Collagen type III alpha chain 1 Reverse	TATACCTGGAAGTCCGGGGG
Laminin subunit beta 1 Forward	TCCGAGACAGGTCACTGCTA
Laminin subunit beta 1 Reverse	TGACTCCGCAAAGCAACTGT
Tissue inhibitor of metallopeptidase Forward	GGAATGCACAGTGTTTCCCTG
Tissue inhibitor of metallopeptidase Reverse	GGAAGCCCTTTTCAGAGCCT
EMC7 Forward	GGGCTGGACAGACTTTCTAATG
EMC7 Reverse	CTCCATTTCCCGTCTCATGTCAG
CHMP2A Forward	CGCTATGTGCGCAAGTTTGT
CHMP2A Reverse	GGGGCAACTTCAGCTGTCTG
C1orf43 Forward	CTATGGGACAGGGGTCTTTGG
C1orf43 Reverse	TTTGGCTGCTGACTGGTGAT

were washed and incubated with a secondary antibody (anti-rabbit IgG, HRP-linked antibody, Cell Signaling) at room temperature for 1 h. All antibodies were diluted in a 1% fat-free skimmed milk and 0.1% TBST solution. Between stages, membranes were washed in 0.1% TBST. Bands were visualized using an HRP detection system (Super Signal West Dura Extended Duration Substrate, Thermo Scientific/Pierce Biotechnology). Chemiluminescence was measured using a ChemiDoc MP System (Bio-Rad Laboratories), and band intensities were calculated with molecular imaging software (Image Lab, Bio-Rad Laboratories). All samples were analyzed in duplicate, and mean values were used for statistical analyses.

To measure ubiquitin protein expression, ∼25 mg of tissue were homogenized in RIPA lysis buffer (Millipore, Temecula, CA, United States) composed of Halt™ protease and phosphatase inhibitor cocktail (Thermo Scientific, Waltham, MA, United States). Samples were centrifuged, and protein concentration of the supernatant was measured (Pierce™ BCA Protein Assay Kit, Thermo Scientific). Equal amounts of protein were boiled in 4 × Laemmli buffer; 20 μg of protein were separated by SDS-PAGE and then transferred to PVDF membranes (Bio-Rad Laboratories, Inc., Hercules, CA, United States) using a semidry *Trans*-Blot Turbo™ device (Bio-Rad). The membranes were blocked using 5% bovine serum albumin (BSA) solution in Tris–buffered saline with 0.1% Tween 20 (TBST) for a period of 1 h. Following this step, they were incubated with primary antibodies for ubiquitin (Santa Cruz Biotechnology) diluted 1:1000 in 5% BSA in TBS-T. The membranes were then washed in TBST and incubated with anti-mouse secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, United States) linked to horseradish peroxidase (1:5000) for 1 h at room temperature. The membranes were exposed on a ChemiDoc image device (Bio-Rad) using enhanced chemiluminescence reagent (ECL Select kit; GE Healthcare Ltd., Little Chalfont, United Kingdom). The staining intensity of the whole lane was measured using ImageJ (NIH, Bethesda, MD, United States), but the bottom of the membrane (∼10 kDa) was excluded to avoid measuring free ubiquitin. Ubiquitinated protein was normalized to GAPDH to ensure equal loading.

Immunohistochemistry Staining

Muscle sections were cut to 10-μm thickness in a cryomicrotome (CM 1860 UV, Leica, Nussloch, Germany) set at −20°C, before mounting the sections on microscope slides (Superfrost Plus, Thermo Scientific, Waltham, MA, United States). Muscle sections were fixed in 2% paraformaldehyde for 5 min followed by 10 min permeabilization in 0.2% triton X-100 in PBS. Sections were then blocked in 2% BSA and 5% goat serum in PBS for 60 min at room temperature. Primary antibodies against tenascin C (MA5-16086, Thermo Scientific, Rockford, IL, United States; 1:100) and dystrophin (Ab15277, Abcam, Cambridge, United Kingdom; 1:500) were diluted in 2% BSA and incubated over night at 4°C. The following day, sections were incubated for 60 min at room temperature with appropriate secondary antibodies (A11001, Invitrogen Molecular Probes, Rockford, IL, United States; CF594, Biotium, Fremont, CA, United States; both 1:200) diluted in the blocking buffer. The sections were then covered with ProLong Gold Antifade Reagent with DAPI (P36935, Invitrogen Molecular Probes) and covered with a cover slip. Between stages, sections were washed three times for 10 min in PBS with 0.05% Tween-20. Images of the muscle tissue sections were captured using a camera (DP72, Olympus, Tokyo, Japan) mounted on a microscope (BX61, Olympus) with a fluorescence light source (X-Cite, 120PCQ, EXFP Photonic Solutions Inc., Mississauga, ON, Canada), using a 10 × 0.30 numerical aperture air objective (UPlanFL N, Olympus). To compare the intensity of staining for tenascin C between samples, settings for the microscope, camera and software were fixed. One image was obtained per section, which usually covered most of the sample. By using Fiji imaging software (Schindelin et al., 2012) an optimal threshold for positive staining was set, and used for all images to display percentage area of positive tenascin C staining.

Statistical Analysis

Prior to statistical analysis, the Shapiro–Wilk test was used to check whether data were normally distributed. Log transformations were applied to data that were not normally distributed. Normally distributed data were analyzed using a 2 × 3 repeated measures ANOVA to calculate time, trial, and time × trial interaction effects. Paired *t*-tests were used to compare changes over time and differences between the trials. Normally distributed data are presented as mean ± SD, whereas log-transformed data are presented as the geometric mean ± 95% confidence interval of the geometric mean. Data that were not normally distributed after log transformation were analyzed using the Friedman's test. When this test revealed a significant result. Wilcoxon's signed ranked tests were used to compare any changes over time and differences between the trials. Non-normally distributed data are presented as mean ± interquartile range. The false discovery rate was used to correct for multiple comparisons. The Statistics Package for Social Sciences version 23 (IBM, Armonk, NY, United States) was used to conduct the statistical analysis. Statistical significance for time, trial, and time × trial interaction main effects was set at $P < 0.05$.

RESULTS

IGF-1 Ec mRNA expression was higher than pre-exercise 24 h (1.7-fold; $P = 0.038$) and 48 h (1.5-fold; $P = 0.038$) after active recovery, and 24 h (2.1-fold; $P = 0.038$) after cold water immersion (**Figure 1**). IGF-1 Ea mRNA expression did not

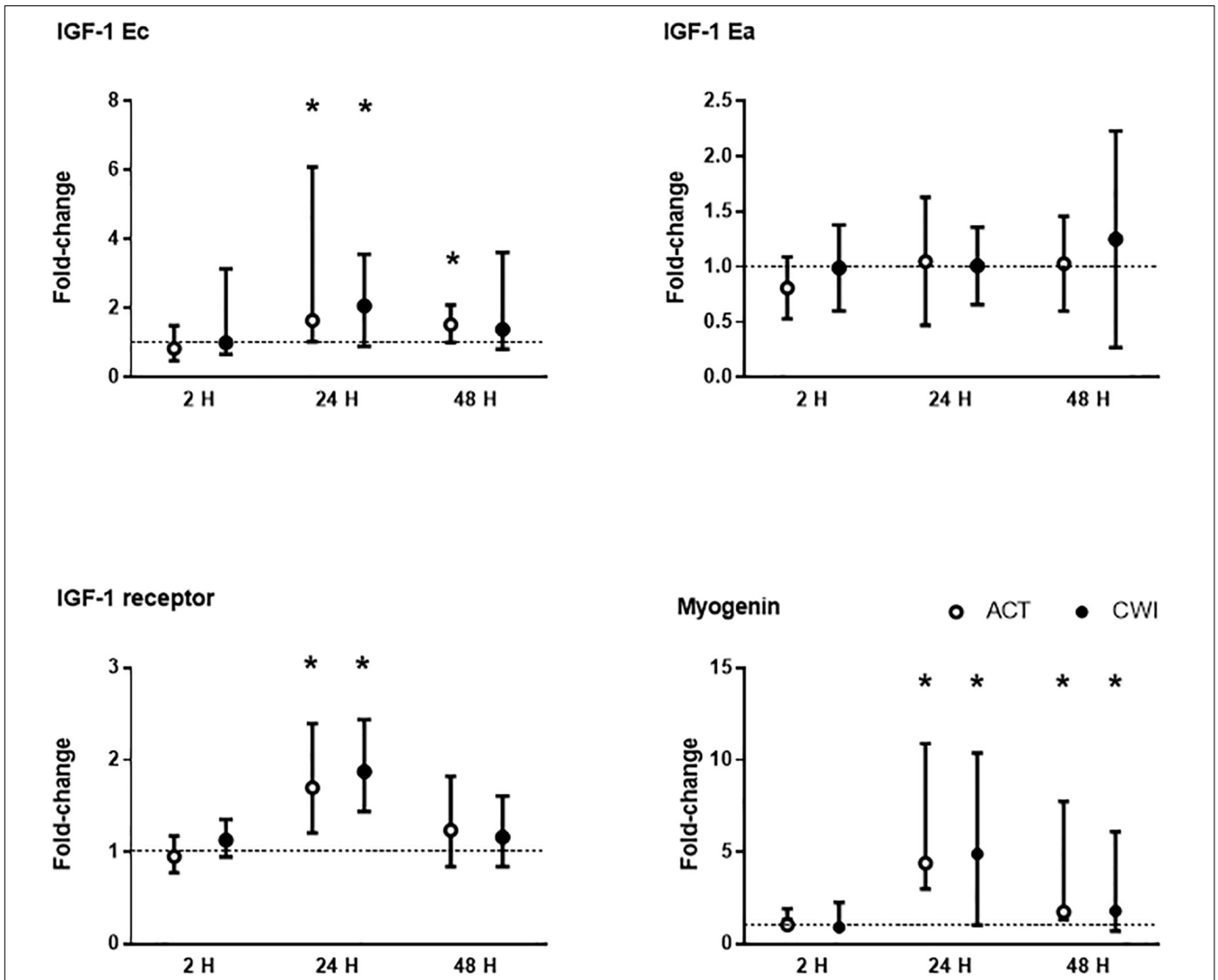

FIGURE 1 | Post-exercise changes in mRNA expression of insulin-like growth factor (IGF)-1 splice variants IGF-1 Ea and IGF-1 Ec (mechano growth factor), IGF-1 receptor and myogenin. Data are presented as changes in the mean ± SD for IGF-1 Ea, and the median ± interquartile range for IGF-1 Ec, IGF-1 receptor and myogenin. *$P < 0.05$ versus pre-exercise. ACT, active recovery; CWI, cold water immersion. The dotted line represents a nominal pre-exercise value of 1.0 (for comparison).

change significantly after either trial (P = 0.55) (**Figure 1**). IGF-1 receptor mRNA expression was higher than pre-exercise 24 h after active recovery (1.7-fold; P = 0.007) and cold water immersion (1.9-fold; P = 0.001) (**Figure 1**). Myogenin mRNA expression was higher than pre-exercise 24 h after active recovery (4.4-fold; P = 0.0039) and cold water immersion (4.9-fold; P = 0.0039), and 48 h after active recovery (1.7-fold; P = 0.0039) and cold water immersion (1.8-fold; P = 0.027). The expression of IGF-1 splice variants, myogenin and IGF-1 receptor did not differ significantly between the trials.

GADD45a mRNA expression was higher than pre-exercise 2 h after active recovery (10.0-fold; $P < 0.001$) and cold water immersion (13.7-fold; $P < 0.001$) (**Figure 2**). It increased further after 24 h, and remained higher than pre-exercise 48 h after both trials. GADD45b mRNA expression was also higher than pre-exercise 2 h after active recovery (18-fold-fold; P = 0.008) and cold water immersion (28-fold; P = 0.008) (**Figure 2**). It remained higher than pre-exercise 24 h after cold water immersion (2.3-fold; P = 0.028). The expression of GADD45a and GADD45b did not differ significantly between the trials.

MuRF-1 mRNA expression was higher than pre-exercise 2 h after active recovery (2.1-fold; P = 0.002) and cold water immersion (2.3-fold; P = 0.008) (**Figure 3**). It was lower than pre-exercise 48 h after active recovery (−5%; P = 0.026). Atrogin-1 mRNA expression was lower than pre-exercise 2 h after active recovery (−12%; P = 0.015) and cold water immersion (−10%; P = 0.012) (**Figure 3**). It decreased further after 24 h, and remained lower than pre-exercise 48 h after both trials. Myostatin mRNA expression did not change significantly after exercise in either trial (**Figure 3**; P = 0.86 for active recovery; P = 0.44 for cold water immersion). The expression of MuRF-1, atrogin-1 and myostatin did not differ significantly between the trials. Ubiquitin protein expression did not change significantly after either trial (P = 0.77 for active recovery; P = 0.17 for cold water immersion).

Collagen type I alpha chain 1 mRNA expression was higher than pre-exercise 24 h after active recovery (2.1-fold; P = 0.004). It was also higher 24 h (1.8-fold; P = 0.003) and 48 h (2.2-fold; P = 0.019) after cold water immersion (**Figure 4**). Laminin subunit beta 1 mRNA expression followed a similar pattern of changes compared with COL1A expression (**Figure 4**). Collagen type III alpha chain 1 mRNA expression was higher than pre-exercise 24 h (1.8-fold; P = 0.008) and 48 h (1.7-fold; P = 0.005) after active recovery, and 24 h (2.3-fold; P = 0.014) and 48 h (2.1-fold; P = 0.007) after cold water immersion (**Figure 4**). Tissue inhibitor of metallopeptidase 1 mRNA expression was higher than pre-exercise 2 h (2.5-fold; P = 0.002), 24 h (9.5-fold; $P < 0.001$ and 48 h (3.4-fold; P = 0.008) after active recovery. It was also higher than pre-exercise 2 h (2.3-fold; P = 0.002), 24 h (8.8-fold; $P < 0.001$) and 48 h (3.8-fold; P = 0.008) after cold water immersion (**Figure 4**). Tenascin C protein expression was higher than pre-exercise 2 h after active recovery (2.5-fold; P = 0.022), whereas it did not change significantly after cold water immersion (**Figure 5**). The expression of ECM genes and tenascin C protein did not differ significantly between the trials.

FoxO3a expression in the cytosolic fraction of muscle homogenates was lower than pre-exercise 2 h (0.3-fold; $P < 0.001$) and 48 h (0.7-fold; P = 0.001) after active recovery.

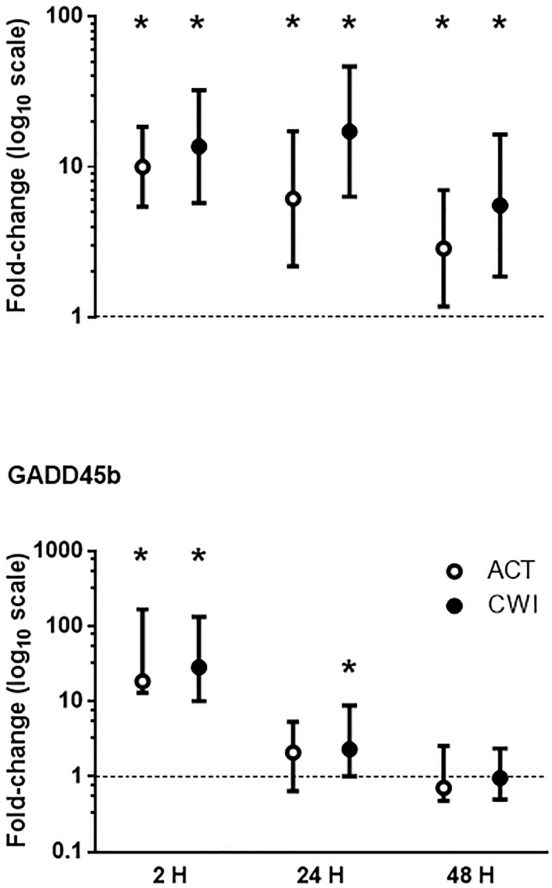

FIGURE 2 | Post-exercise changes in mRNA expression of growth arrest and DNA damage-inducible protein alpha (GADD45a) and GADD45 beta (GADD45b). Data are presented as changes in the geometric mean ± 95% confidence interval of the geometric mean for GADD45A, and the median ± interquartile range for GADD45B. *$P < 0.05$ versus pre-exercise. ACT, active recovery; CWI, cold water immersion. The dotted line represents a nominal pre-exercise value of 1.0 (for comparison).

It was also lower than pre-exercise 2 h (0.2-fold; $P < 0.001$), 24 h (0.4-fold; P = 0.005) and 48 h (0.4-fold; P = 0.007) after cold water immersion (**Figure 6**). FoxO3a expression in the nuclear fraction of muscle homogenates was lower 2 h after active recovery (0.5-fold; P = 0.029) and cold water immersion (0.3-fold; P = 0.001) (**Figure 6**). The expression of FoxO3a did not differ significantly between the trials.

DISCUSSION

The present study aimed to compare how cold water immersion and active recovery after acute resistance exercise influence molecular factors that regulate muscle growth and remodeling. Resistance exercise induced the expression of the growth factors IGF-1 Ec and GADD45, the E3 ubiquitin ligase muscle-specific

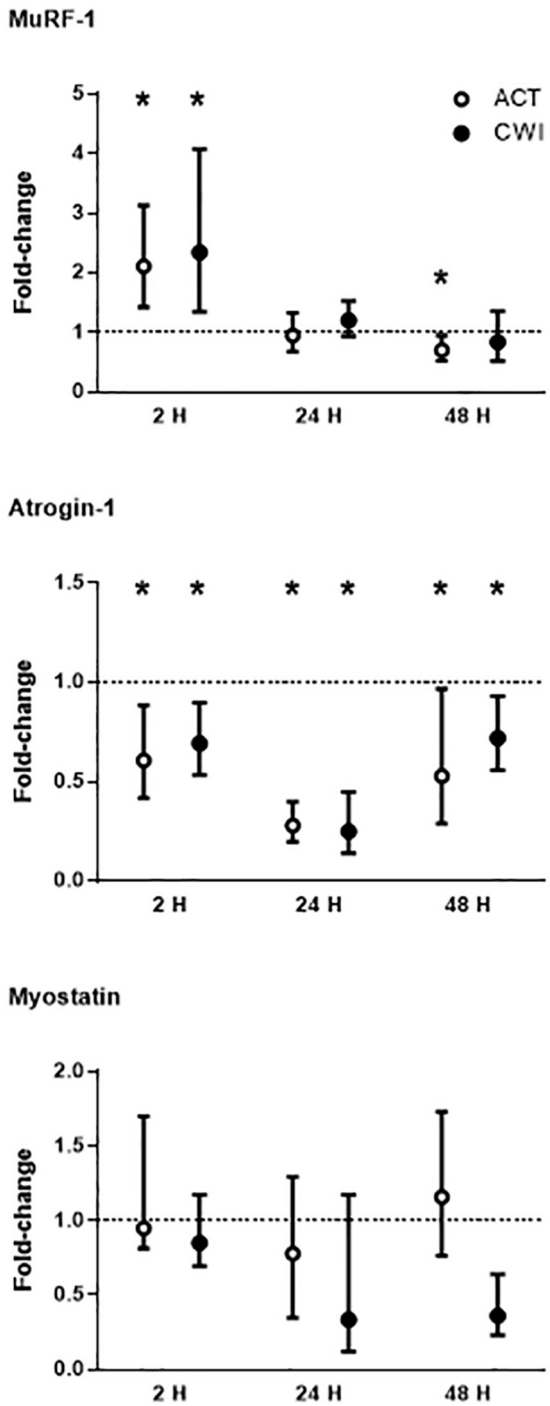

FIGURE 3 | Post-exercise changes in mRNA expression of muscle-specific RING finger 1 (MuRF-1), atrogin-1 and myostatin. Data are presented as the median ± interquartile range. *$P < 0.05$ versus pre-exercise. ACT, active recovery; CWI, cold water immersion. The dotted line represents a nominal pre-exercise value of 1.0 (for comparison).

RING finger 1, and various factors involved in ECM remodeling (i.e., collagen type I alpha chain 1, collagen type III alpha chain 1, laminin subunit beta 1, tissue inhibitor of metallopeptidase 1). However, these responses were not significantly different between the cold water immersion and active recovery treatments. The present findings suggest that regular cold water immersion attenuates muscle hypertrophy (Roberts et al., 2015; Fyfe et al., 2019) independently of changes in factors that regulate myogenesis, proteolysis and extracellular matrix remodeling in muscle after exercise.

Consistent with other research (Psilander et al., 2003; McKay et al., 2008; Wilborn et al., 2009; Heinemeier et al., 2013) we found that mRNA expression of IGF-1 receptor and IGF-1 Ec (also known as mechano growth factor) increased in muscle, whereas IGF-1 Ea did not change significantly after resistance exercise (**Figure 1**). IGF-1 splice variants are proposed to regulate activation, proliferation and differentiation of muscle cells (McKay et al., 2008). We did not find any significant differences in the expression of IGF-1 Ec or IGF-1 receptor mRNA after cold water immersion compared with active recovery. It is unlikely that cold water immersion attenuates chronic muscle adaptation to strength training (Roberts et al., 2015; Fyfe et al., 2019) by reducing IGF-1 signaling pathways in muscle. Our findings contrast with two animals studies reporting that icing either attenuated IGF-1 mRNA expression (Vieira Ramos et al., 2016) or delayed IGF-1 protein expression (Takagi et al., 2011) in muscle after injury. This disparity may reflect differences between the effects of cold water immersion versus icing and the time course of molecular responses in muscle to resistance exercise versus severe muscle trauma.

We also measured mRNA expression of the growth factor GADD45 in muscle after exercise. GADD45 is a small myonuclear protein that translocates to the nucleus of muscle cells in response to cell stress. Here, it modifies myonuclear morphology and activates extensive alterations in the expression of genes that regulate the balance between muscle growth and atrophy (Ebert et al., 2012). GADD45A mRNA remained elevated for the entire recovery period after exercise, while GADD45B mRNA peaked 2 h after exercise (**Figure 2**), which supports previous research (Chen et al., 2003; Mahoney et al., 2008). GADD45 mRNA expression was not significantly different between the cold water immersion and active recovery trials. The exercise-induced increase in GADD45 in muscle after exercise is intriguing, because it seems to play a dual role in muscle. GADD45 activates many genes associated with muscle atrophy (Ebert et al., 2012). Conversely, it is upregulated in rat muscle following overload-induced hypertrophy (Carson et al., 2002). Also, transfection with plasmids encoding GADD45 in mouse muscle increases myogenin expression (Bongers et al., 2013). GADD45 most likely plays a role in muscle remodeling, but this role may differ under conditions of muscle disuse compared with mechanical loading, including exercise. GADD45 is regulated by histone deacetylase 4 (HDAC4) (Bongers et al., 2013), activating transcription factor 4 (ATF4; also known as CREB2), FoxO and p53 (Kastan et al., 1992; Tran et al., 2002; Ebert et al., 2012). Among these factors, HDAC4 (Russell et al., 2013), FoxO and p53 (Stefanetti et al., 2014) [but not ATF4 (Ogborn et al., 2014)] are activated in muscle after exercise, and may have induced GADD45 mRNA expression in muscle following exercise. Based on the present findings, it would seem that cold water immersion

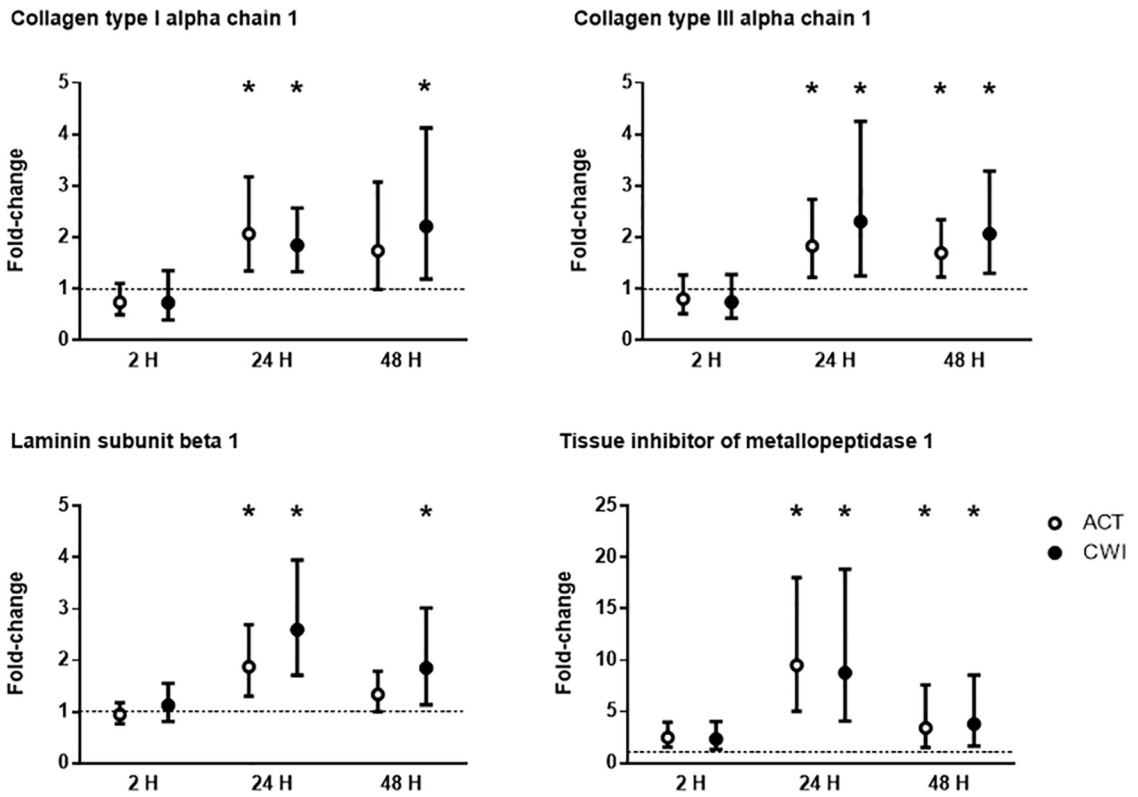

FIGURE 4 | Post-exercise changes in mRNA expression of collagen type I alpha chain 1, collagen type III alpha chain 1, laminin subunit beta 1 and tissue inhibitor of metallopeptidase 1. Data are presented as changes in the median ± interquartile range. *$P < 0.05$ versus pre-exercise. ACT, active recovery; CWI, cold water immersion. The dotted line represents a nominal pre-exercise value of 1.0 (for comparison).

did not influence any of these factors that control GADD45 expression in muscle.

Icing delays muscle regeneration following muscle injury in rats (Takagi et al., 2011) raising the possibility that cryotherapy may activate signaling pathways that inhibit muscle growth, such as the ubiquitin−proteasome pathway. We found that mRNA expression of muscle-specific RING finger-1 increased, whereas atrogin-1 mRNA expression decreased in muscle following resistance exercise (**Figure 3**). These findings are consistent with other research (Louis et al., 2007; Drummond et al., 2008; Mascher et al., 2008; Manini et al., 2011; Reitelseder et al., 2014; Stefanetti et al., 2014; Zak et al., 2018; Fuchs et al., 2020). However, cold water immersion did not alter the expression of either of these atrogenes. Fuchs et al. also reported no effects of cold water immersion on muscle-specific RING finger-1 and atrogin-1 mRNA expression in muscle between 0 and 5 h after resistance exercise (Fuchs et al., 2020). When comparing the effects of applying cold versus hot thermal pads to muscle after resistance exercise, Zak et al. (2018) observed no significant difference in the expression of these two atrogenes between treatments. It therefore appears unlikely that these ubiquitin-ligases inhibit muscle hypertrophy following repeated cold water immersion (Roberts et al., 2015; Fyfe et al., 2019).

Considering that muscle-specific RING finger-1 and atrogin-1 both promote muscle atrophy (Kang et al., 2017), it is interesting that muscle-specific RING finger-1 increases, whereas atrogin-1 decreases in muscle following resistance exercise. The specific factors that regulate the expression of these two ubiquitin-ligases in muscle during exercise remain to be determined. Likely candidates include FoxO3 (Kang et al., 2017), TNF associated factor (TRAF)-6 (Paul et al., 2012) and the TNF weak inducer of apoptosis (TWEAK)−fibroblast growth factor-inducible 14 (Fn14) system (Hindi et al., 2014). Variation in exercise-induced expression of some of these regulatory factors (Reitelseder et al., 2014; Stefanetti et al., 2014; Raue et al., 2015) may account for the divergent changes in the expression of muscle-specific RING finger-1 and atrogin-1 after exercise that we and others have observed. Muscle-specific RING finger-1 likely influences muscle remodeling after exercise by interacting with (and controlling) the half-life of structural proteins such as troponin, myosin light and heavy chains, and myosin binding protein C (Schiaffino et al., 2013). Atrogin-1 interacts with sarcomeric proteins, the intermediate filaments desmin and vimentin, transcriptional factors, metabolic enzymes and components of translation (Schiaffino et al., 2013). Downregulation of atrogin-1 mRNA expression may result in less degradation of these proteins in muscle after exercise.

In contrast with other research reporting a decrease in myostatin mRNA expression in muscle following resistance

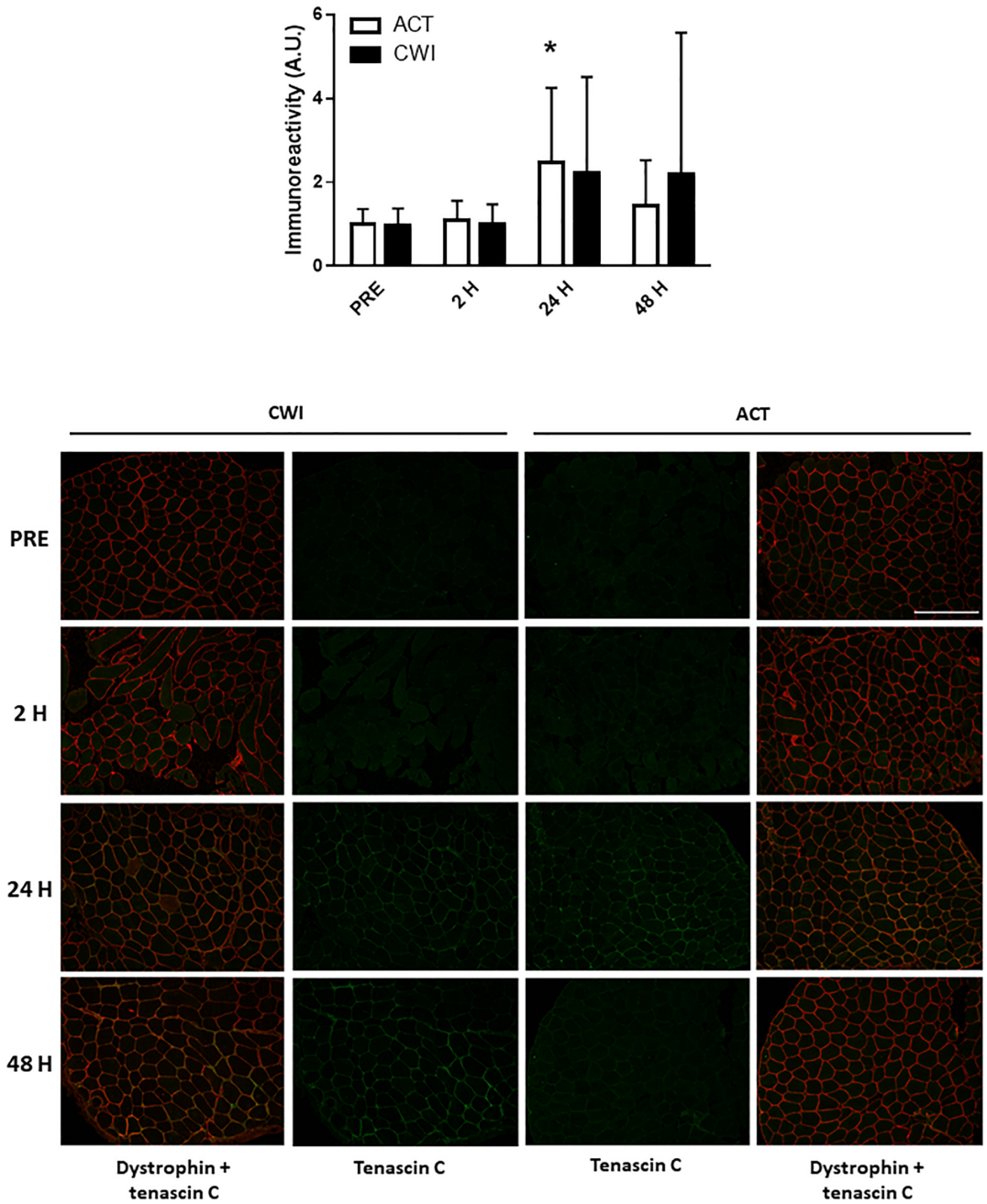

FIGURE 5 | Post-exercise changes in expression tenascin C protein and representative images of tenascin C immunoreactivity in skeletal muscle of one participant. Data are presented as the mean ± SD. *$P < 0.05$ versus pre-exercise. ACT, active recovery; CWI, cold water immersion. Scale bar represents 200 μm.

exercise (Louis et al., 2007; Deldicque et al., 2008; Drummond et al., 2008; Mascher et al., 2008; Wilborn et al., 2009; Zak et al., 2018), we observed no significant change in myostatin. Myostatin expression in skeletal muscle cells depends (in part) on the expression or activity of follistatin (Lee et al., 2010). The exercise protocol in the present study may not have been sufficiently intense to induce follistatin expression in muscle (Laurentino et al., 2012) and thereby suppress myostatin mRNA expression. Another factor that could account for variation in myostatin responses to exercise is differences in the myostatin splice variants that have been assessed in each study. Interactions between splice variants can influence the expression and activity of myostatin in muscle cells (Shin et al., 2015). There were no differences in mRNA expression of muscle-specific RING finger-1, atrogin-1 or myostatin in response to cold water immersion compared with active recovery.

FoxO proteins are a group of transcription factors that regulate various cellular functions in muscle including energy

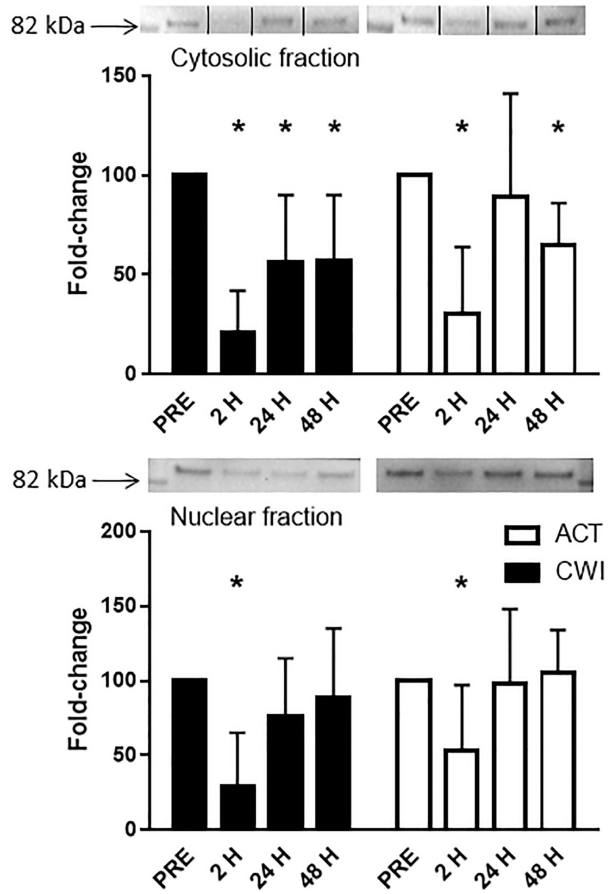

FIGURE 6 | Representative blots and data for expression of FoxO3a in the cytosolic fraction and nuclear fraction isolated from muscle tissue homogenates. Western blotting for FoxO3a in the cytosolic fraction was performed simultaneously on the same membrane for another protein (data published elsewhere (Peake et al., 2017). For the purpose of clarity here, the images for the blots have been cropped (indicated by the lines on the blot) to show only the blots for FoxO3a. Data are expressed as mean ± SD. *$P < 0.05$ versus pre-exercise. ACT, active recovery; CWI, cold water immersion.

homeostasis, mitochondrial metabolism, protein breakdown, cell cycle, apoptosis and muscle regeneration (Sanchez et al., 2014). Other research has reported no effect of cold water immersion on gene expression of FoxO1 in muscle after resistance exercise (Fuchs et al., 2020). At the protein level, Fyfe et al. observed that cold water immersion did not alter the expression of phosphorylated FoxO3a^{Ser253} in muscle after resistance exercise (Fyfe et al., 2019). We found that FoxO3a protein levels in cytosolic and nuclear fractions of muscle decreased after exercise, with no significant difference between cold water immersion and active recovery (**Figure 6**). To our knowledge, this is the first study to compare changes in the protein abundance of FoxO3a in separate subcellular fractions in skeletal muscle after exercise. Others have reported a similar decrease in FoxO3a protein in cytoplasmic and nuclear fractions of C2C12 myotubes treated with IGF-1 (Tong et al., 2009). Tong et al. (2009) also found that IGF-1 increased phosphorylation of FoxO3a at Thr$^{318/321}$ in the cytoplasm. They proposed that in concert with Akt, IGF-1 sequesters and phosphorylates FoxO3a in the cytoplasm, thereby attenuating or blocking ubiquitin−ligase activity (Sandri et al., 2004; Stitt et al., 2004). We did not measure the expression of Akt or phosphorylated FoxO3, so unfortunately, we cannot confirm whether these molecular interactions are also in play in muscle tissue after resistance exercise. It is possible that FoxO is more active and translocates to the nucleus during exercise, whereas after exercise Akt and IGF-1 may suppress this subcellular movement. Regardless, the present findings suggest that cold water immersion did not influence any regulatory factors upstream of FoxO3, such as the IGF-1−PI3K−Akt pathway. Partial support for this notion is that we observed no significant differences in IGF-1 Ec mRNA expression between the trials (**Figure 1**).

Icing after crush injury increases the proportion of collagen fibers in the extensor digitorum muscle of rats (Takagi et al., 2011). By contrast, freeze injury reduces mRNA expression of collagen I, collagen III and connective tissue growth factor, whereas it does not alter the proportion of collagen fibers in the *tibialis anterior* muscle of mice (Vieira Ramos et al., 2016). To date, no research has investigated the effects of postexercise cooling on ECM remodeling in muscle after resistance exercise. ECM remodeling is an important component of muscle repair and growth. Specifically, in damaged muscle, the ECM provides a scaffold for satellite cells and other stromal cells (e.g., macrophages fibroblasts, endothelial cells, pericytes) to interact with each other to form new muscle fibers (Mackey and Kjaer, 2017). We observed that mRNA expression of collagen type I alpha chain 1, collagen type III alpha chain 1, laminin and tissue inhibitor of metallopeptidase 1 increased in muscle following resistance exercise (**Figure 4**), which supports the work of others (Crameri et al., 2004, 2007; Mikkelsen et al., 2011; Hoier et al., 2012; Heinemeier et al., 2013; Hyldahl et al., 2015). All of these factors contribute to ECM remodeling, although their individual roles in the reconstruction of myofibers and their matrix requires further research (Mackey and Kjaer, 2017). Staining for tenascin C in muscle also increased after exercise (**Figure 5**). In the context of ECM remodeling, tenascin-C is believed to disassemble focal adhesion complexes between cells and the matrix, thereby assisting cell survival, motility and tissue repair (Mackey and Kjaer, 2017). The expression of ECM genes and tenascin-C was similar between the cold water immersion and active recovery trials. These findings suggest that the smaller gains in muscle mass and strength after repeated cold water immersion (Roberts et al., 2015) are not likely a result of impaired ECM remodeling.

The strengths of this study include the randomized, crossover design, the "real-world" resistance training protocol, the inclusion of participants who were training regularly, the pre- and postexercise control procedures, and the breadth of molecular analysis. A limitation is that we did not collect muscle biopsies more than 48 h after exercise. The muscle biopsy procedure is invasive, and the participants in this study provided eight biopsy samples in total. Adding another biopsy sample more than 48 h after exercise would have added to the burden on the participants. Two other studies have reported that mRNA expression of IGF-1, myogenin, atrogin-1 and myostatin in muscle had returned to

pre-exercise values by 72−96 h after exercise (Bickel et al., 2005; Deldicque et al., 2008). By contrast, another study reported that mRNA expression of IGF-1Ea, IGF-1Ec, and myogenin remained elevated between 72 and 120 h after exercise (McKay et al., 2008). The time course of changes in factors that regulate muscle growth and remodeling after exercise is therefore variable, and may depend on factors such as the nature of preceding exercise. Another limitation is that we did not include a loading control when performing the Western blots for FoxO3a protein expression. We are confident in our results, however, because we measured total protein concentration in triplicate before loading samples onto the gels, and each sample was analyzed in duplicate.

In summary, although athletes often immerse their body in cold water after intense exercise to expedite return to training and competition, evidence continues to accumulate that when used regularly, this practice can attenuate long-term gains in muscle mass and strength. In the present study, resistance exercise induced robust alterations in various genes and proteins involved in myogenesis, proteolysis and extracellular remodeling in muscle. However, cold water immersion after exercise did not alter the expression of these genes and proteins in muscle between 2 and 48 h after exercise. Putting the present findings into context with other research, regular cold water immersion appears to attenuate adaptations to strength training by suppressing the expression of genes and proteins involved in processes or pathways other than myogenesis, proteolysis and extracellular remodeling.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Human Research Ethics Committee of the University of Queensland. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JP contributed to the conception, design and interpretation of data for this work, drafted the work, and provided approval for publication of the content. JM contributed to acquisition of data for this work and provided approval for publication of the content. KC, SA, and VF contributed to acquisition of data for this work, drafted the work, and provided approval for publication of the content. LR contributed to the conception and design for this work, and provided approval for publication of the content. TR and DC-S contributed to the conception and design for this work, revised it critically for important intellectual content, and provided approval for publication of the content. All authors contributed to the article and approved the submitted version.

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Macrophage Subpopulations and the Acute Inflammatory Response of Elderly Human Skeletal Muscle to Physiological Resistance Exercise

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The current model for repair of damaged tissue includes immune cells, mediating the progression from a pro-inflammatory to an anti-inflammatory environment. How this process changes with aging in human skeletal muscle under conditions of physiological exercise loading remains unclear. To investigate this, 25 elderly males (mean age 70 ± SD 7 years), as well as 12 young (23 ± 3 years) and 12 elderly (74 ± 3 years) females, performed a unilateral bout of heavy resistance leg extension exercise. Biopsies were collected from the vastus lateralis muscle of the rested (control) leg, and post exercise from the exercised leg at 4.5 h, and on days 1, 4, and 7 for the male participants, or on day 5 for the female participants. Total macrophages (CD68+) as well as pro- (CD11b+) and anti-inflammatory (CD163+, CD206+) subpopulations were identified on sections by immunohistochemistry. Gene expression levels of COL1A1, TNF-a, CD68, myostatin, TCF7L2, IL-1B, IL-1R, IL-10, and Ki67 were determined by real-time RT-PCR. At rest, the muscle tissue from the elderly vs. young females was characterized by higher gene expression levels of CD68, IL-10, lower myostatin mRNA, and trends for a greater number of macrophages, while COL1A1 mRNA post exercise values were greater in the elderly vs young. For the male participants, mRNA levels of the inflammatory cytokines IL-1B, IL-1R were elevated in the early phase following exercise, followed by increases in COL1A1 and Ki67 on days 4 and 7. In general, exercise induced increases in all types of macrophages counted in the elderly, but not in young, individuals. Cells expressing CD68, CD11b, and CD206 simultaneously were the most frequently observed cell type, which raises the possibility that pure pro- and anti-inflammatory macrophages populations do not exist in healthy human skeletal muscle within the spectrum of tissue remodeling induced by physiological exercise designed to induce hypertrophy. Together these data provide insight into the time course of macrophage activity and associated molecular targets in human skeletal muscle in the context of aging and exercise.

Keywords: macrophages, human skeletal muscle, resistance exercise, sarcopenia, COL1A1, CD68, CD11b, CD206

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INTRODUCTION

Skeletal muscle is a plastic tissue with a significant capacity to regenerate and adapt following severe damage, even in elderly individuals in their 60s (Karlsen et al., 2020). The developing model for repair of damaged tissue includes immune cells, mediating the progression from a pro-inflammatory response to an anti-inflammatory state (Arnold et al., 2007; Tidball and Villalta, 2010; Saclier et al., 2013a). This process is precisely regulated and is crucial for proper regeneration, as evidenced in studies perturbing the infiltration sequence to later identify altered and impaired muscle regeneration (Chazaud, 2014). However, it appears that a certain degree of dysregulation of the inflammatory response exists in elderly individuals (Reidy et al., 2019) which could be a potential factor in the age-related loss of skeletal muscle mass. Furthermore, in patients with peripheral artery disease, low macrophage content of the gastrocnemius muscle was found to be associated with better walking performance (Kosmac et al., 2020) suggestive of potential clinical implications for macrophages in human skeletal muscle.

Following muscle damage, resident macrophages are believed to orchestrate the attraction of monocytes to the site of injury (Brigitte et al., 2010) where the local environment will drive the monocyte toward a macrophage phenotype (Kumar and Jack, 2006; Murray and Wynn, 2012). Macrophage phenotypes are generally classified into two opposing categories: (1) pro-inflammatory macrophages (M1) containing phagocytic properties, enhanced microbicidal capacity and a high secretion of pro-inflammatory cytokines (IL-1B and TNF-a), or (2) anti-inflammatory macrophages (M2) associated with anti-apoptosis, tissue repair and growth factors (Mosser and Edwards, 2008). These characteristics are mostly described from *in vitro* studies and likely represent the extremes in a spectrum of many possible phenotypes (Saclier et al., 2013b). Cytokines expressed by macrophages have also been shown to regulate myogenic precursor cell fate (Arnold et al., 2007; Saclier et al., 2013a; Chazaud, 2014) further supporting a role for macrophages as key players in muscle regeneration, repair and maintenance.

Muscle regeneration, as defined by events following myonecrosis (Grounds, 2014) is, however, an extreme state of tissue remodeling, compared to the remodeling induced by typical physiological exercise regimens performed by individuals throughout the lifespan. Most information about macrophages has been elucidated from very invasive animal studies and *in vitro* studies, and indeed severe eccentric protocols in humans (Jones et al., 1986; Paulsen et al., 2010a,b, 2012a) as reviewed earlier (Paulsen et al., 2012b; Peake et al., 2017). However, there is relatively little knowledge about macrophage infiltration in human skeletal muscle under physiological settings and even fewer studies comparing young and elderly individuals directly. For example, it has been shown that 14 weeks of progressive heavy resistance training induces increases in the number of anti-inflammatory and total number of macrophages in the skeletal muscle of elderly individuals (Walton et al., 2019a). Similarly, in a group of individuals ranging in age from 29 to 68, 12 weeks of endurance cycle training was observed to increase the anti-inflammatory macrophage content of the working muscles, where resident anti-inflammatory muscle macrophages are associated with exercise-mediated increases in skeletal muscle fiber size, suggesting a role in muscle growth (Walton et al., 2019b). Of the few studies comparing young and elderly subjects, recent work has investigated local macrophage content using a muscle damage protocol and found infiltration sequences mimicking the pattern of that seen in animal studies, and also showing differences between young and elderly subjects (Sorensen et al., 2019). This supports the findings from an earlier study where cellular and molecular differences between young and elderly individuals were observed 3 days after a physiological bout of resistance exercise (Przybyla et al., 2006). However, a detailed time-course for inflammatory cell infiltration and the associated molecular response following a physiological bout of resistance exercise remains yet undescribed.

Furthermore, there is a general lack of consensus regarding appropriate markers to distinguish between pro- and anti-inflammatory macrophages on sections of human skeletal muscle. A recent study addressed this methodological issue, proposing an immunohistochemistry protocol, validated by flow cytometry, While CD68 is considered a macrophage pan marker and CD11b has been used as a marker for proinflammatory macrophages (Przybyla et al., 2006; Sorensen et al., 2019) a study by Kosmac et al. (2018) reported that CD68 and CD11b can be used interchangeably as pan markers highlighting the need for continued research and development into methods identifying markers and their specificity to immune cells. This complexity is important to recognize when designing antibody-based protocols. The study by Kosmac also found that the most commonly observed cell type was positive for both CD11b and CD206 and that macrophages in human skeletal muscle in the rested state simultaneously express markers of the so-called classic pro-inflammatory and anti-inflammatory phenotypes (Kosmac et al., 2018) indicating that the current classification may not reflect the true *in vivo* state in healthy human skeletal muscle. Building on these studies, and to address the paucity of knowledge on the time course of the macrophage response in young and elderly healthy individuals, the main purpose of this study was to compare the inflammatory profile of young and elderly skeletal muscle at rest and in response to a single bout of physiological resistance exercise. We hypothesized that, when compared to younger individuals, the skeletal muscle of elderly individuals would be characterized by a greater presence of anti-inflammatory macrophages, in accordance with earlier work (Sorensen et al., 2019) reflected by a corresponding gene expression profile. Furthermore, we hypothesized that the macrophage response to exercise would be dominated by anti-inflammatory macrophages in the elderly, but not young, participants.

MATERIALS AND METHODS

Experimental Design

This study is based on muscle biopsy analyses from two studies, one on 25 elderly men (Heisterberg et al., 2018) and one on 12 young and 12 elderly women (Bechshøft et al., 2019;

Soendenbroe et al., 2020). Both studies were approved by The Committees on Health Research Ethics for The Capital Region of Denmark (Ref: H-15017223, H-3-2012-081). All procedures conformed to the Declaration of Helsinki and the subjects gave written informed consent before participation. Generally, all participants in both studies were considered healthy, non-smokers and did not perform any strenuous physical exercise on a regular basis – more information about the participants is available in **Table 1**. Initially, the men were part of a randomized controlled trial investigating the effect of the blood pressure lowering medication Losartan on the muscle response to exercise in healthy normotensive males, where half of the participants received Losartan and the other half placebo. Given the general lack of any drug effect (Heisterberg et al., 2018) the two groups in the male study were merged in the present study (separate group data are also provided for reference in **Supplementary Material**).

For both studies, the exercise protocol consisted of one session of unilateral knee extension heavy resistance exercise. The exercised leg was chosen by randomization and the inactive leg served as a control leg. The heavy resistance exercise consisted of a concentric and eccentric exercise bout where load was determined from one repetition max (RM) performed in a leg-extension machine (M52, TechnoGym, Cesena, Italy). The exercise was performed using the same equipment as described in detail (Heisterberg et al., 2018; Bechshøft et al., 2019). Briefly, the men performed 5 sets of 12 concentric repetitions (70% 1RM) followed by 4 sets of 6 eccentric repetitions (110% 1 RM). The exercises were performed with a two-minute break between the sets and five-minute break between the two different types of exercise. The women performed 4 sets of 12 repetitions (70% 1RM) of concentric contractions with a two-minute break between each set. This bout was followed by 4 sets of 4 repetitions (110% 1RM) of eccentric contractions with two-minute breaks between each set. After 5–10-min of resting the women performed the entire protocol a second time.

Muscle Biopsies

For all participants, muscle biopsies were obtained from the vastus lateralis muscle, under local anesthetic (1% lidocaine), using the percutaneous needle biopsy technique of Bergström, with 5–6 mm needles and manual suction. Pieces of muscle tissue were aligned, embedded in Tissue-Tek, and frozen in isopentane, pre-cooled in liquid nitrogen, and stored at −80°C. The men had six muscle biopsies taken over 17 days, at the following time points: −10 and −3 days before exercise (from the control, non-exercised, leg), and from the exercised leg at +4.5 h, and on days +1, +4, and +7 post exercise. The day −3 sample was excluded from the current study since its purpose was to investigate a potential effect of losartan in the rested state and is therefore irrelevant in the current context. The young and elderly women had muscle biopsies collected from each leg 5 days after exercise.

TABLE 1 | Participant characteristics.

	Young females		Elderly females		Elderly males	
	n = 12		*n = 12*		*n = 25*	
Age (yr)	23 ± 3	[20–28]	74 ± 3	[70–78]	70 ± 7	[64–90]
Height (cm)	168 ± 7	[157–177]	165 ± 3	[159–169]	180 ± 5	[172–189]
Weight (kg)	64 ± 8	[53–75]	68 ± 10	[57–84]	82 ± 10	[67–98]
BMI (kg/m^2)	23 ± 2	[19–26]	25 ± 4	[20–30]	26 ± 3	[21–31]
Knee extension 1RM (kg)	39 ± 8	[30–50]	24 ± 5	[12–29]	56 ± 14	[23–82]

RNA-Extraction

Approximately 2–5 mg of muscle tissue (100 cryosections of 10 μm) was homogenized in 1 ml of TriReagent (Molecular Research Center, Cincinnati, OH, United States) containing five stainless-steel balls of 2.3 mm in diameter (BioSpec Products, Bartlesville, OK, United States) by shaking in a FastPrep-24 instrument (MP Biomedicals, Illkirch, France) at speed level 4 for 15 s. Following homogenization, bromo-chloropropane was added to separate the samples into an aqueous phase and an organic phase. Following isolation of the aqueous phase, RNA was precipitated using isopropanol. The RNA pellet was then washed in ethanol and subsequently dissolved in 20 μl of RNAse-free water. Total RNA concentrations and purity were determined by spectroscopy at 260, 280, and 240 nm. Good RNA integrity was ensured by gel electrophoresis.

Real-Time RT-PCR

Five hundred nanogram of total RNA was converted into cDNA in 20 μl using the OmniScript reverse transcriptase (Qiagen) and 1 μM poly-dT (Invitrogen, Naerum, Denmark) according to the manufacture's protocol (Qiagen). For each target mRNA, 0.25 μl of cDNA was amplified in a 25-μl SYBR Green polymerase chain reaction (PCR) containing 1 Quantitect SYBR Green Master Mix (Qiagen) and 100 nM of each primer (**Table 2**). The amplification was monitored in real time using the MX3005P Realtime PCR machine (Stratagene). The CT values were related to a standard curve made with known concentrations of cloned PCR products or DNA oligonucleotides (Ultramer oligos; Integrated DNA Technologies, Leuven, Belgium), with a DNA sequence corresponding to the sequence of the expected PCR product. The specificity of the PCR products was confirmed by melting curve analysis after amplification. RPLP0 mRNA was chosen as internal control. To validate this use, another unrelated "constitutive" mRNA, GAPDH, was measured and normalized with RPLP0 (Heisterberg et al., 2018; Soendenbroe et al., 2020).

Immunohistochemistry

Sections (10 μm) of the muscle biopsies were cut at −20°C in a cryostat and placed on glass slides. In the male study immunohistochemical labeling was performed using primary antibodies CD68 and CD163 (**Table 3**). Briefly, sections were fixed in 5% formaldehyde (Histofix; Histolab, Gothenburg, Sweden) for 10 minutes and refrigerated overnight with 2 primary antibodies in 1% BSA buffer, according to **Table 3**. The following day 2 secondary antibodies (**Table 3**) diluted in 1% BSA were added for 45 min. Finally, slides were mounted with Prolong-Gold-Antifade with DAPI (Cat. No. P36931; Molecular Probes/Invitrogen). Between all steps, slides were washed in two

TABLE 2 | PCR primers.

mRNA	NCBI ID	Sense	Anti-sense
RPLP0	NM_053275.3	GGAAACTCTGCATTCTCGCTTCCT	CCAGGACTCGTTTGTACCCGTTG
GAPDH	NM_002046.4	CCTCCTGCACCACCAACTGCTT	GAGGGGCCATCCACAGTCTTCT
COL1A1	NM_000088.3	GGCAACAGCCGCTTCACCTAC	GCGGGAGGACTTGGTGGTTTT
TNFa	NM_000594.3	TTCCCCAGGGACCTCTCTCTAATC	GAGGGTTTGCTACAACATGGGCTAC
CD68	NM_001251.2	CAGCTTTGGATTCATGCAGGACC	CTCTGCCCCAGGGGTGCTTG
Myostatin	NM_005259.2	TGCTGTAACCTTCCCAGGACCA	GCTCATCACAGTCAAGACCAAAATCC
Myogenin	NM_002479.5	CTGCAGTCCAGAGTGGGGCAGT	CTGTAGGGTCAGCCGTGAGCAG
TCF7L2	NM_001146274.1	CGGAAGGAGCGACAGCTTCAT	GTCTCTCCCGGCTGCTTGTCC
IL-1B	NM_000576.2	TGCGTGTTGAAAGATGATAAGCCCA	CAAATCGCTTTTCCATCTTCTTCTTTG
IL-10	NM_000572.3	CGCTGTCATCGATTTCTTCCCTGT	TGGCTTTGTAGATGCCTTTCTCTTGG
IL-1R	NM_000877.4	GGAAGGGATGACTACGTTGGGGA	CCAGCCAGCTGAAGCCTGATGTT
Ki67	NM_002417.4	CGGAAGAGCTGAACAGCAACGA	GCGTCTGGAGCGCAGGGATA

TABLE 3 | Antibodies.

Males				
Host	**Antibody**	**Company**	**Cat. no.**	**Concentration**
Primary antibody				
Rabbit	CD68	Sigma-Aldrich	HPA048982	1:200
Mouse	CD163	Santa Cruz	SC-2006	1:200
Host		**Company**	**Cat. no.**	**Concentration**
Secondary antibody				
Goat Anti-rabbit 568		Thermo Scientific	A-11036	1:500
Goat anti-mouse 488		Thermo Scientific	A-1109	1:500
Females				
Host	**Antibody**	**Company**	**Cat. no.**	**Concentration**
Primary antibody				
Mouse	CD68	Dako	M0718	1:500
Rabbit	CD11b	Abcam	AB52478	1:500
Goat	CD206	RD Systems	AF2534	1:1000
Host		**Company**	**Cat. no.**	**Concentration**
Secondary antibody				
Donkey anti mouse 488		Abcam	Ab-150109	1:200
Donkey anti rabbit 568		Abcam	Ab-175693	1:200
Donkey anti goat 680		Thermo Scientific	A-21084	1:500

changes of 0.05M Tris-buffered Saline (TBS). Slides were stored at room temperature for 24 h, protected from sunlight, and then stored at −20°C until analysis by microscopy. For the samples in the female study the same method was followed but developed further to include three primary antibodies. In order to have three antibodies from three different host species, CD206 was used instead of CD163, as supported elsewhere (Kosmac et al., 2018, 2020) resulting in the following three primary antibodies: CD68, CD11b, and CD206 (**Table 3**). Other changes to the protocol were that samples were blocked with 5% donkey serum in TBS for 30 min and then refrigerated overnight with three primary antibodies diluted in 1% donkey serum in TBS, according to **Table 3**. The following day 3 secondary antibodies (**Table 3**) diluted in 1% donkey serum were added for 60 min, before slides were mounted.

Microscopy

Images from each section were collected using a DP71 Olympus Camera mounted on a BX51 Olympus microscope (x20/0.50NA objective) using the Olympus cellSens software (v.1.14). Image size was 2040 × 1536 pixels, corresponding to 868 × 653 μm. For all sections, the goal was to image areas where transversely aligned fibers filled the entire image, avoiding perimysium where possible since it usually contains higher numbers of macrophages than the endomysium. These areas, along with longitudinally oriented fibers and sectioning artifacts were excluded from the analysis.

The same person conducted image analysis for all participants. Blinded to time, the following sequence of analysis was performed in the male study: number of fibers and area of tissue included in the analysis, cells that were (1) CD68+CD163−, (2) CD68+CD163+, and (3) CD68−CD163+. Each cell was evaluated manually in ImageJ (v.150c4, National Institutes of Health, United States). Cells were only marked positive for a marker if the cell nucleus (DAPI) was centrally located within the area of antibody staining. CD68+ was considered a pan-macrophage marker while CD68+CD163+ cells were considered anti-inflammatory. In **Figure 3** CD68+ total is a combination of CD68+CD163− and CD68+CD163+ cells. Pro-inflammatory macrophages were not identified in the male study.

In the female study, blinded to age and exercise, the following sequence of analysis was performed in addition to fiber count and tissue area: cells that were only positive for either CD68, CD11b or CD206, cells that were CD68+CD11b−CD206+, CD68+CD11b+CD206−, CD68−CD11b+CD206+ (double positive), and cells that were CD68+CD11b+CD206+ (triple positive). **Figures 1**, **2** show examples of all identified cell categories. CD68 was considered a pan-macrophage marker, while co-localization with CD11b+ or CD206+ was considered pro-inflammatory and anti-inflammatory, respectively. For comparison with other studies, pro (CD68+CD11b+) and anti-inflammatory macrophages (CD68+CD206+) are displayed in addition to CD68+CD11b+CD206+ in **Figures 5B,C**.

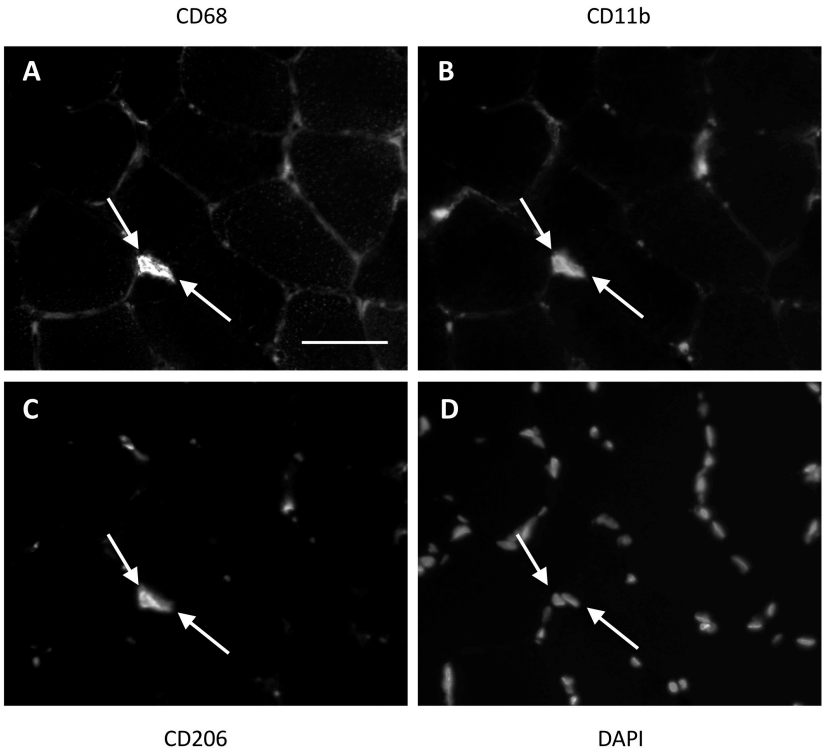


FIGURE 1 | Method for identification of macrophages in the female study. Two triple positive cells (indicated with white arrows) located next to each other in a muscle section. CD68 **(A)** was used as a pan-macrophage marker, CD11b **(B)** as a proinflammatory phenotype marker and CD206 **(C)** as an anti-inflammatory phenotype marker. DAPI **(D)** identifies the cell nucleus. Cells were only marked positive for a marker if the cell nucleus was centrally located within an area of antibody staining. Scale bar indicates a length of 50 μm.

Table 4 provides additional information on how cell counts have been classified and combined in the female study. Some macrophage populations were observed in very limited numbers in the male study (CD68−CD163+) and female study (CD68−CD11b+CD206+, CD68−CD11b−CD206+), so these data are not reported.

All macrophage data are expressed relative to the number of fibers included in each section, or the tissue area.

Statistics

All figures were prepared in GraphPad Prism (v.7.04, GraphPad Software, Inc., La Jolla, CA, United States) and all statistical analyses were conducted in SigmaPlot (v. 13.0, Systat Software Inc, San Jose, CA, United States), except subject characteristics and gene expression of the female subjects, which was analyzed by *t*-test in Microsoft Excel 2016 (Microsoft Corporation, Redmond, WA, United States), and the Spearman rank correlations, which were performed in GraphPad Prism. *P*-values below 0.05 were considered significant. For both studies, mRNA data were normalized to RPLP0 and log-transformed before statistical analysis. For the male participants, since cell count data were not normally distributed, Friedman Repeated Measures Analysis of Variance on ranks was used, while mRNA data were analyzed using a one-way repeated measures analysis of variance. Dunnett's *post hoc* test for multiple comparisons was used to compare each time point with baseline (−10 days). Cell count data from the male study are displayed as individual values (cells/fiber) while mRNA data are displayed as geometric mean ± back transformed SEM. The cell count data from the female study were analyzed by Wilcoxon signed rank test to compare data within the same group (trained vs. untrained), while Mann–Whitney Rank sum test was used to compare between groups (young vs. old) in the control leg only. *P*-values were then Bonferroni corrected using a multiplication factor of 3 which is the number of comparisons made. Cell counts are displayed as individual data (cells/fiber). For the female participants, unpaired *t*-tests were performed between young and old for mRNA data while paired *t*-tests were conducted for the mRNA analysis of the exercise response (exercised leg vs. control leg). As for the males, female mRNA data are displayed as geometric mean ± back transformed SEM. Subject characteristics and number of fibers analyzed are presented as means with standard deviation and range.

RESULTS

Immunohistochemistry – Male

Figure 3 illustrates changes in muscle CD68+ cell content/fiber over time after an acute bout of resistance training. PRE

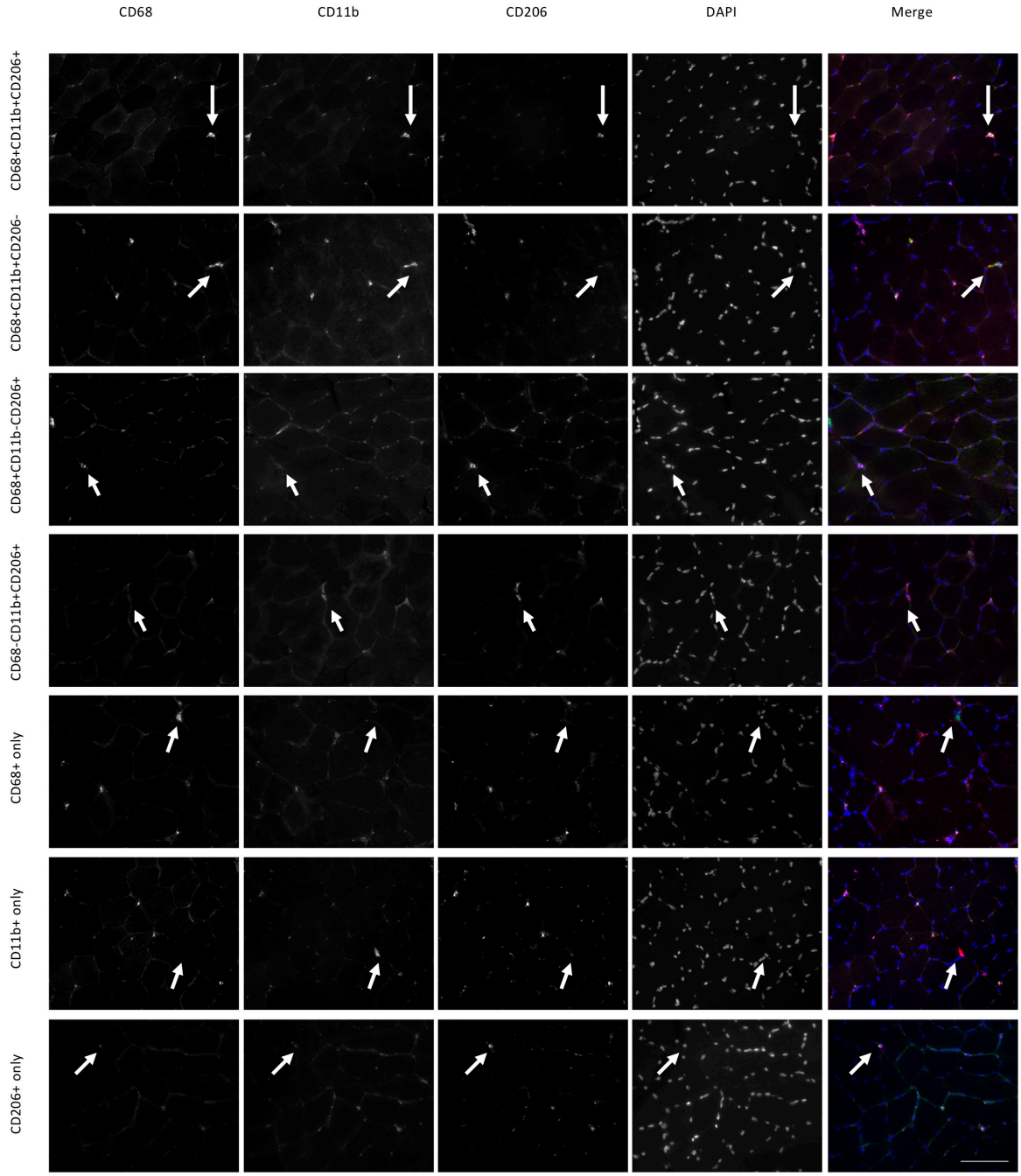


FIGURE 2 | Macrophage histology and identified cell categories in the female study. Each row is a collection of images showing the same area of a muscle section. Using three antibodies (CD68, CD11b, CD206) we identified seven categories of labeled cells, each represented on a different row. For each category white arrows point to the same cell. Scale bar indicates a length of 100 μm.

values from subjects (0.048 cells/fiber) increased on D4 (0.075 cells/fiber, 56%, $P = 0.04$) and D7 (0.090 cells/fiber, 88%, $P < 0.001$), with 3 participants peaking on D1, 7 on D4, 14 on D7, and 1 participant failing to show any increase. Data for CD68+CD163+ cells show an increase from PRE (0.024 cells/fiber) to D7 (0.066 cells/fiber, 175%, $P = 0.002$). Generally, the same pattern were seen for data expressed as cells/mm^2. On average, the number of fibers included in the macrophage analysis was 212 ± 58 [98–403].

mRNA – Male

Data are presented in **Figure 4**. Most of the measured genes demonstrated changes over the 7-day time-course. Compared to pre values gene expression for CD68 was upregulated at 4.5H

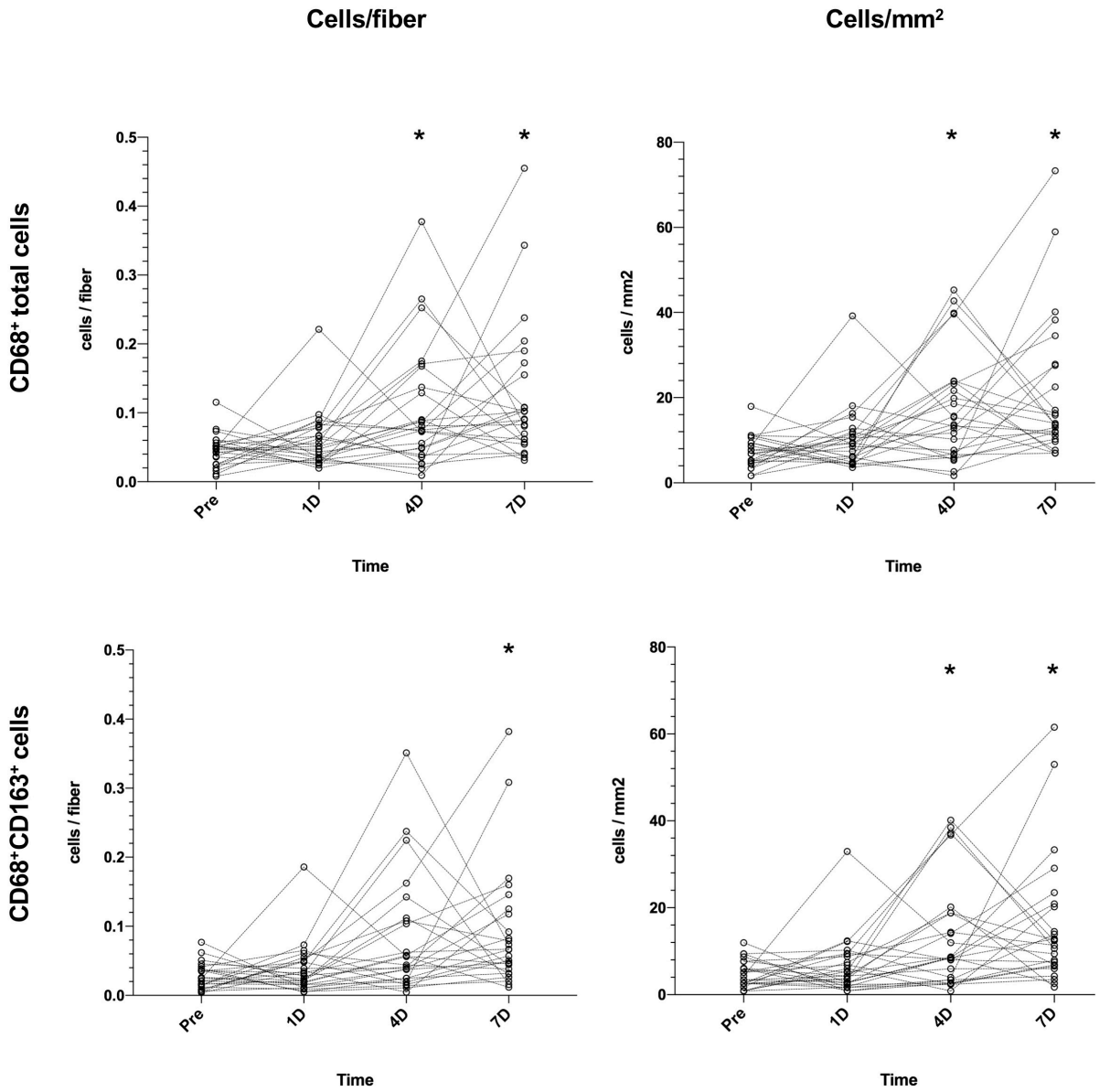


FIGURE 3 | Changes in muscle CD68+ cells **(top)** and CD68+CD163+ cells **(bottom)** in healthy elderly men (n = 25) over a 7-day time course following an acute bout of heavy resistance leg extension exercise. Data was analyzed using Friedman repeated measures analysis of variance on ranks and each timepoint was compared with baseline (–10 days) using Dunnett's *post hoc* test. Individual values are displayed as cells/fiber (left) and cells/mm^2 (right). *$P < 0.05$.

(1.8-fold, $P = 0.001$), D1 (1.9-fold, $P < 0.001$) and D7 (1.6-fold, $P = 0.008$). Other targets related to the inflammatory response showed an acute increase peaking at 4.5H, then returning to, or dropping below, baseline values. This was evident for IL-1B (4.5H, 3.0-fold, $P < 0.001$) and IL-1R (4.5H, 4.4-fold, $P < 0.001$). No changes were detected for TNF-a and IL-10 over the entire time course. Other targets displayed a somewhat delayed response that could not be detected until D4 and D7. This was the case for COL1A1 (D4, 2.0-fold, $P = 0.006$; D7, 2.4-fold, $P < 0.001$) and Ki67 (D4, 2.5-fold, $P = 0.007$; D7, 3.2-fold, $P < 0.001$). Gene expression for Myostatin was down regulated following the exercise session both at 4.5H (0.6-fold, $P = 0.002$), D1 (0.5-fold, $P < 0.001$) and D4 (0.6-fold, $P = 0.005$). Other targets that were down regulated were GAPDH on D1 (0.7-fold, $P < 0.001$) and D4 (0.8-fold, $P < 0.001$) and TCF7L2 on D1 (0.6-fold, $P = 0.007$) D4 (0.7-fold, $P = 0.018$) and D7 (0.7-fold, $P = 0.047$).

Immunohistochemistry – Female

Data from the female macrophage analysis are shown in **Figure 5**. The exercised leg in the old group showed an greater number of CD68+ total cells compared to the control leg 5 days after a heavy resistance exercise bout (0.068 vs. 0.088, 29%, $P = 0.021$). When comparing the exercise and control leg in the old group,

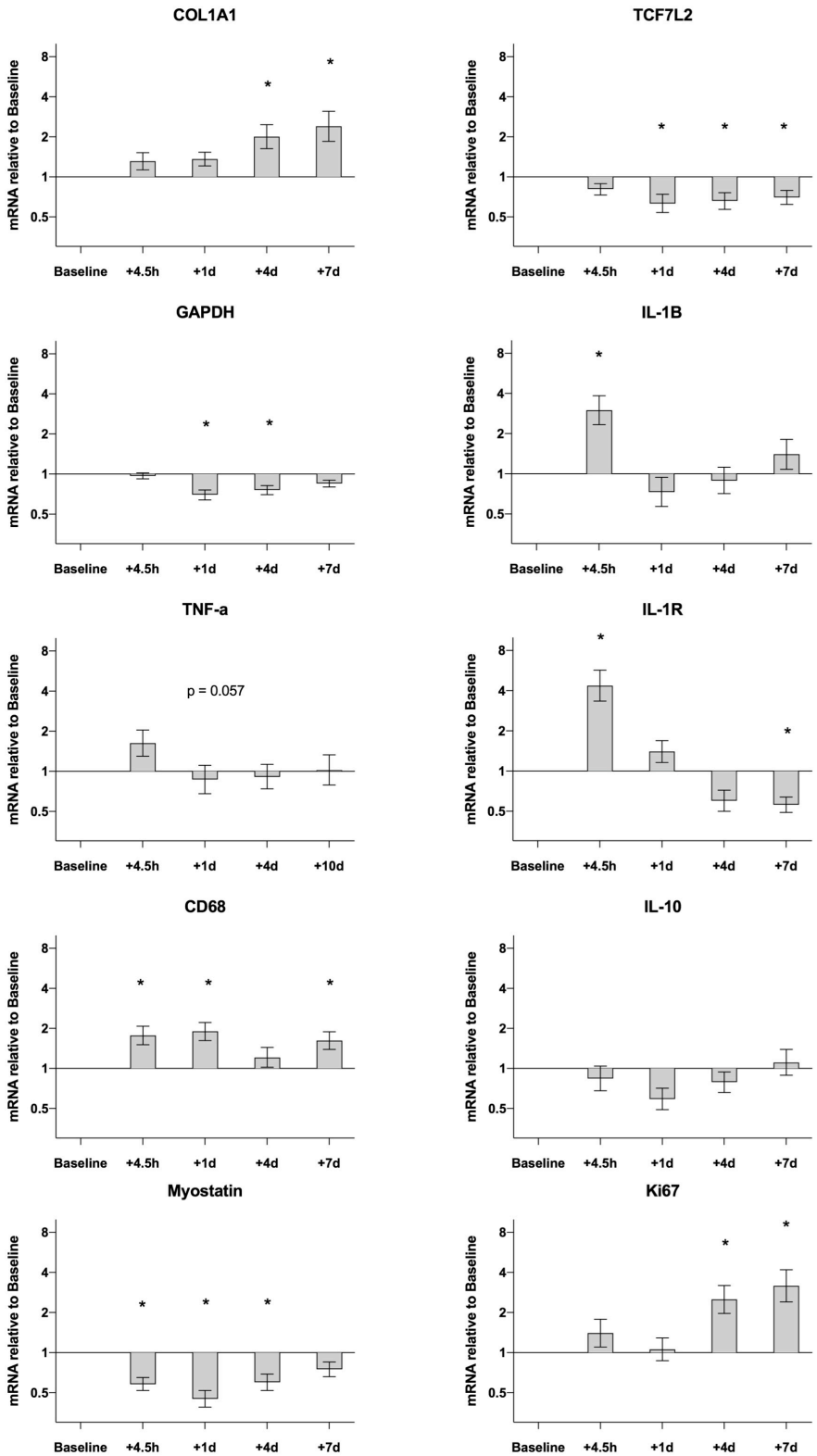


FIGURE 4 | Gene expression in muscle biopsies of healthy elderly men (n = 25) following an acute bout of heavy resistance leg extension exercise. mRNA data were normalized to RPLP0 and are shown as geometric mean ± back transformed SEM, relative to baseline (–10 days). Data were analyzed by one-way repeated measure analysis of variance using Dunnett's *post hoc* test. *$P < 0.05$. Data are Losartan and Placebo groups merged as shown in **Supplementary Figure S2**. Separate group data for COL1A1, Myostatin, GAPDH, CD68 and TCF7L2 have been published previously (Heisterberg et al., 2018).

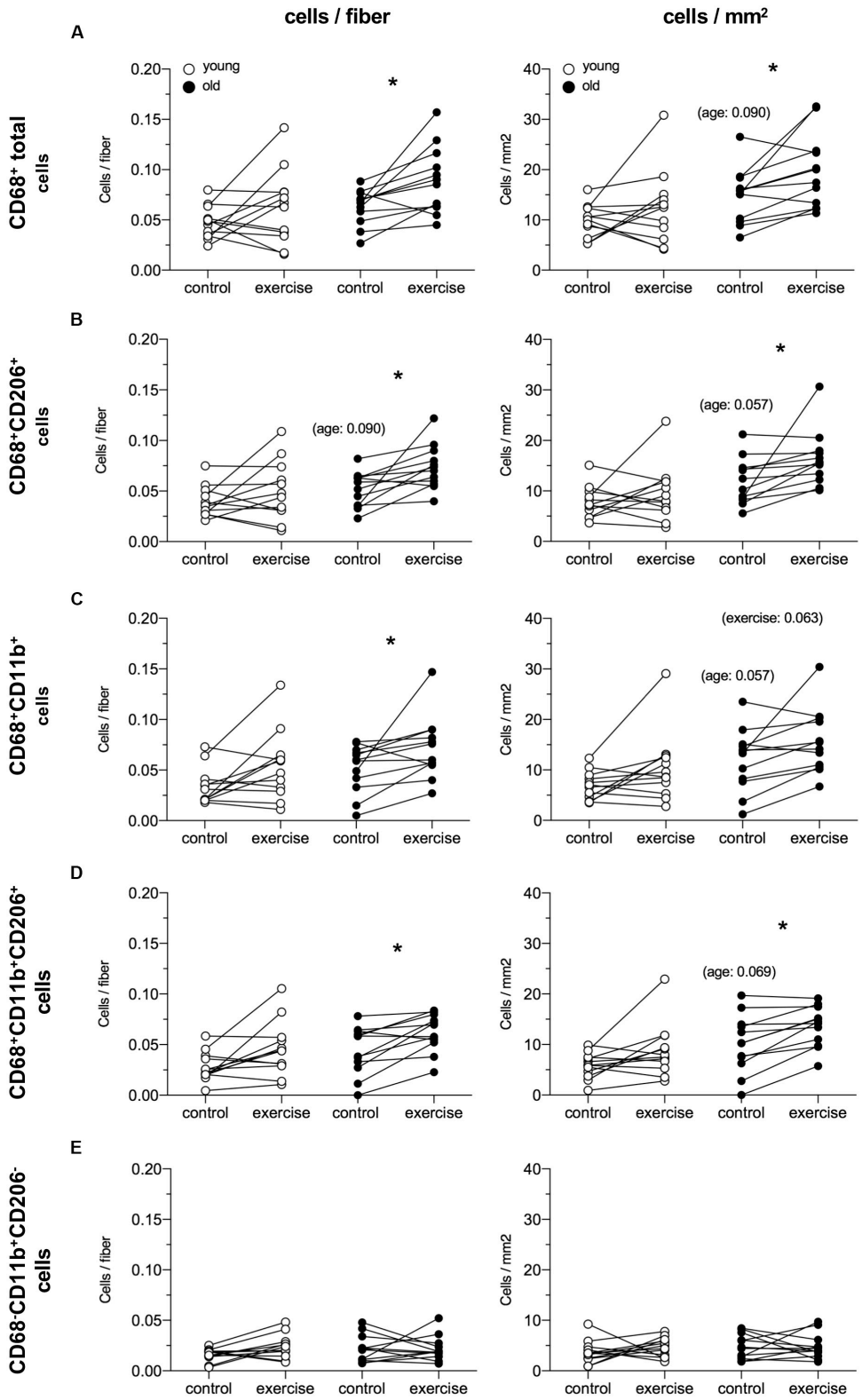


FIGURE 5 | Local macrophage content in healthy young (n = 12) and elderly (n = 12) women from control and exercised leg 5 days following an acute bout of unilateral resistance exercise. Data are presented as cells/fiber (left) and cells/mm^2 (right) for CD68+ total cells **(A)**, CD68+CD206+ cells **(B)**, CD68+CD11b+ **(C)**, CD68+CD11b+CD206+ cells **(D)** and CD68-CD11b+CD206- cells **(E)**. Individual data points are displayed. Data were analyzed using Mann–Whitney (young vs. old) and Wilcoxon signed rank test (trained vs. untrained), and p-values were Bonferroni corrected (x3). *$P < 0.05$ vs. control leg. The inserted p-values indicate trends.

TABLE 4 | Cell definition table – female.

Definition	Remark	Subpopulations combined
$CD68^+$ total	All cells positive for CD68	$CD68^+$ $CD11b^-$ $CD206^-$ $CD68^+$ $CD11b^+$ $CD206^-$ $CD68^+$ $CD11b^-$ $CD206^+$ $CD68^+$ $CD11b^+$ $CD206^+$
$CD68^+$ $CD11b^+$	Pro-inflammatory macrophages	$CD68^+$ $CD11b^+$ $CD206^-$ $CD68^+$ $CD11b^+$ $CD206^+$
$CD68^+$ $CD206^+$	Anti-inflammatory macrophages	$CD68^+$ $CD11b^-$ $CD206^+$ $CD68^+$ $CD11b^+$ $CD206^+$
$CD68^+$ $CD11b^+$ $CD206^+$	Triple positive cells	$CD68^+$ $CD11b^+$ $CD206^+$
$CD11b^+$ only	Positive for only CD11b	$CD68^-$ $CD11b^+$ $CD206^-$

differences were observed for CD68+CD206+ cells (0.055 vs. 0.072 cells/fiber, 31%, $P = 0.009$), CD68+CD11b+ cells (0.059 vs. 0.068 cells/fiber, 15%, $P = 0.048$) and CD68+CD11b+CD206+ cells (0.048 vs. 0.064 cells/fiber, 33%, $P = 0.021$). No significant differences were observed in the young group. For all cell types, a similar pattern was seen for data expressed as cells/mm^2, except for CD68+CD11b+ cells where only a trend for a change was exercise was seen in the elderly individuals. Furthermore, trends were detected for greater numbers of all macrophage types, except for CD68−CD11b+CD206−, when expressed per mm^2 tissue, in the elderly vs. young control leg. The average number of fibers included in the macrophage analysis was 294 ± 74 [189–422] and 294 ± 88 [146–446] for the young and old group, respectively.

mRNA – Female

Data from the female gene expression analysis are displayed in **Figure 6**. Elevated levels of CD68 (2.0-fold, $P = 0.005$) and IL-10 (3.2-fold, $P < 0.001$) were found in the old group compared to the young. Additionally, Myostatin (0.6-fold, $P = 0.007$) and GAPDH (0.6-fold, $P < 0.001$) levels were significantly lower in the old group, while no significant differences were observed for other targets. Post exercise, greater levels of COL1A1 were observed in the elderly vs. young individuals. Aside from this no significant differences were observed for any of the analyzed targets.

Macrophages Correlations With BMI and 1RM

Significant Spearman correlations were detected between BMI and the number of CD68+ macrophages/fiber in the control leg of the young females, and a trend was detected in the elderly females (**Figure 7**). This was not confirmed for the males, although removal of a potential outlier (0.11 CD68+ cells/fiber) resulted in a similar trend ($r = 0.373$, p-value 0.073) to that observed in the females. No significant relationship was detected between 1RM and the number of CD68+ cells/fiber in any of the groups.

DISCUSSION

The main findings of this study are gradually increasing numbers of macrophages and their subpopulations in response to a single bout of physiological exercise in the skeletal muscle of healthy elderly males and females, in accordance with changes in gene expression levels of inflammatory and collagen targets during the hours and days post exercise. These changes occurred from an altered local muscle inflammatory profile at baseline in elderly vs. young individuals, which together provides novel insights into the inflammatory state of aging human skeletal muscle, both at rest and when challenged with physiological muscle loading. Furthermore, we report for the first time, that the most commonly observed macrophage cell type was positive for all three markers of the macrophage markers CD68, CD11b, and CD206, suggesting that, within the physiological range of exercise-induced perturbations to healthy skeletal muscle, pure pro- or anti-inflammatory macrophages do not exist.

Studies have demonstrated that uninjured muscle in elderly individuals contains a predominance of anti-inflammatory macrophages (Cui et al., 2019; Reidy et al., 2019) which has been associated with the development of fibrosis (Mann et al., 2011) although it is possible that macrophage content increases to combat fibrosis rather than contributing to it. Indeed it has been shown that resident anti-inflammatory muscle macrophages are associated with exercise−mediated increases in skeletal muscle fiber size, suggesting a role in muscle growth (Walton et al., 2019a). Additionally a study found that anti-inflammatory macrophages in elderly are more prevalent than in young subjects prior to, and following, a muscle damaging protocol (Sorensen et al., 2019). In support of this, we detected higher levels of IL-10 and CD68 gene expression in the muscle of the control leg in the elderly compared to young females. It should also be noted that without the Bonferroni correction applied to our immunohistochemistry data, a higher content of anti-inflammatory cells (CD68+CD206+) and total CD68+ cells would have been reported in the control muscle of the elderly compared to young females, underlining that our data are not in contrast with those of Sorensen et al. (2019).

The most consistent outcome of our studies was that in the time frame of 4–7 days post exercise, we observed a clear increase in all macrophage populations in a group of 25 elderly males and 11 elderly females. This was true for both the total macrophage number, defined as CD68+ total, as well as for anti-inflammatory macrophages, defined as being either CD68+CD163+ or CD68+CD206+. The issue of molecular markers to define macrophages as pro- or anti-inflammatory is unresolved. Recently this was addressed, where it was observed that the majority of macrophages on cryo-sections of human muscle were positive for both CD11b and CD206 (Kosmac et al., 2018). Our findings are in line with this and, due to the triple labeling approached used in the current study, we add the novel insight that the most prevalent macrophage type observed was positive for CD11b, CD206, and CD68, outnumbering cells expressing CD11b or CD206 in combination with CD68. However, the findings of the majority of macrophages in human muscle

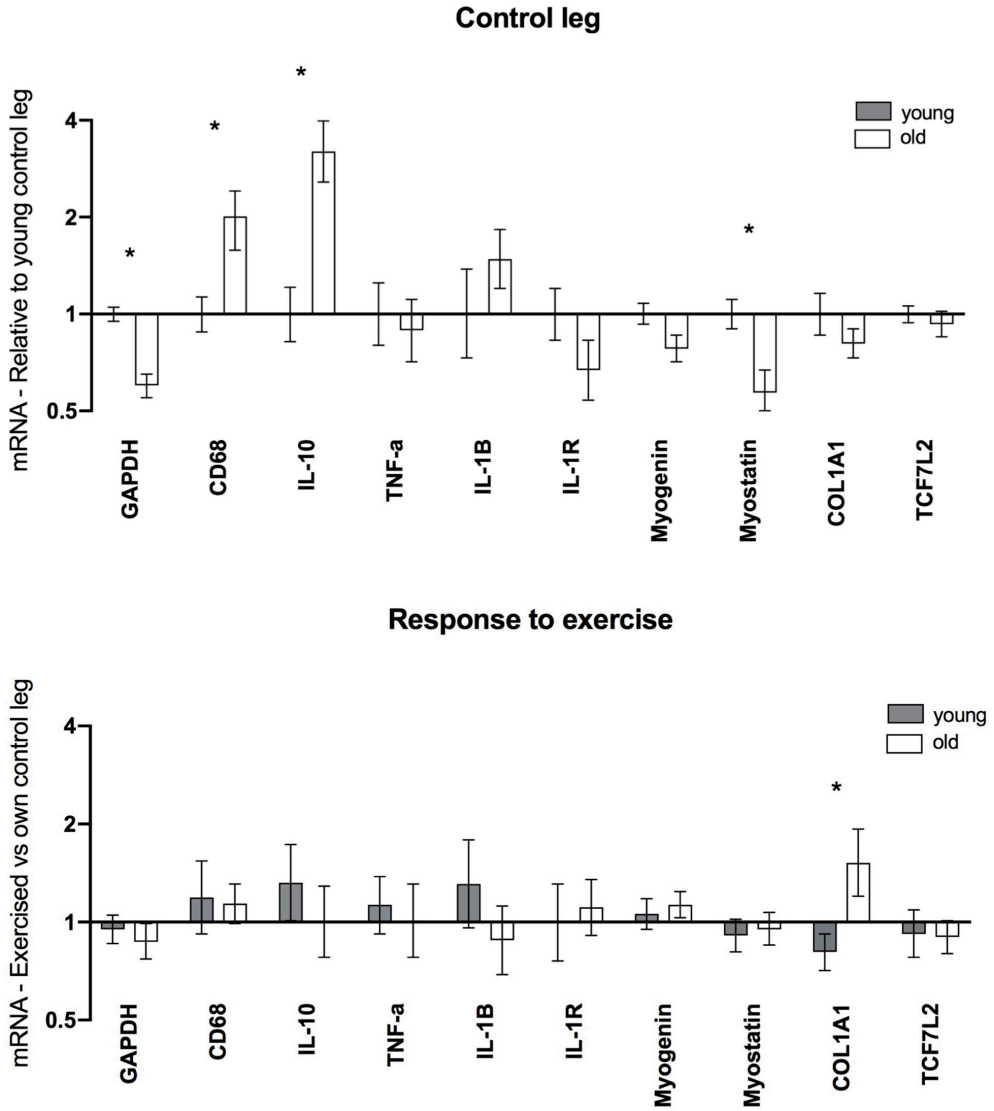
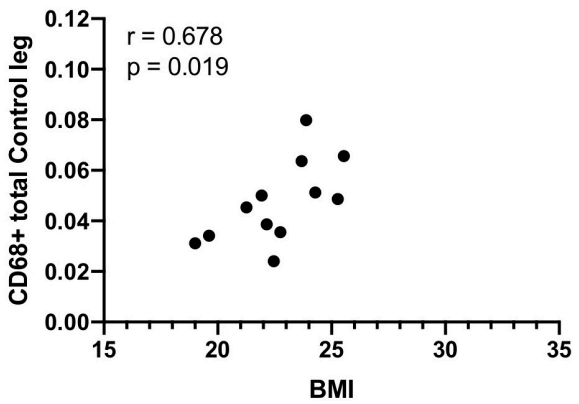
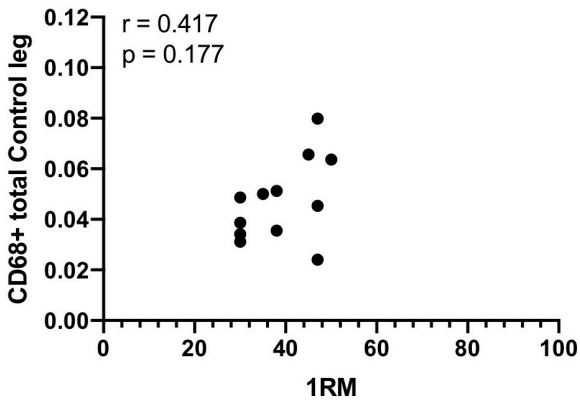


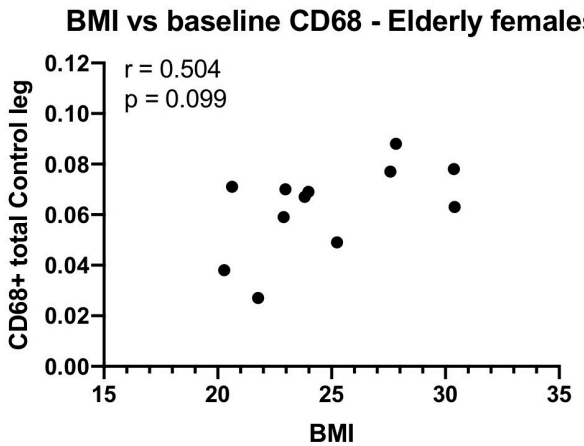
FIGURE 6 | Gene expression in muscle biopsies of healthy young (n = 12) and elderly (n = 12) women 5 days after a single bout of unilateral resistance exercise. mRNA data were normalized to RPLP0 and shown as geometric mean ± back transformed SEM, relative to young control leg (Control leg) and own control leg (Response to exercise). *$P < 0.05$ vs. young. GAPDH data have been published previously (Soendenbroe et al., 2020).

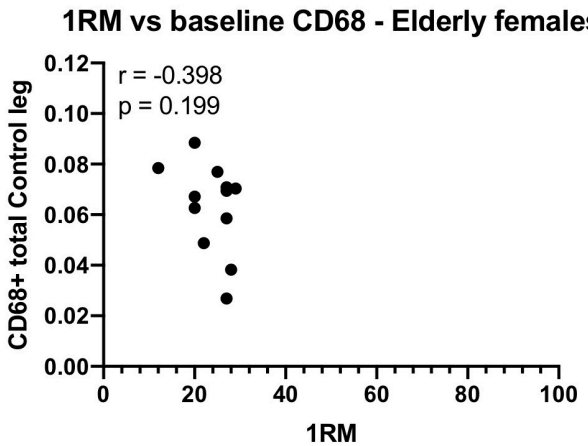
being positive for both CD11b and CD206 (Kosmac et al., 2018) strongly brings into question the use of CD11b as a useful pro-inflammatory marker. Our analyses were initiated before this recent publication, where CD11b was still considered a pro-inflammatory marker, but this may be inaccurate. Similarly, our switching between CD163 and CD206 to identify anti-inflammatory macrophages in the two sets of samples was due to practical issues of needing the three primary antibodies to be from three different host species, rather than a theoretical reasoning that one was better than the other. Indeed it has been shown that the two correlate well in human muscle (Kosmac et al., 2020) so this is unlikely to make a major difference. Reconciling the various macrophage markers, and interpretation of their use, in the literature as a whole is challenging. Currently it would appear that anti-inflammatory macrophages can be identified with more confidence than pro-inflammatory macrophages, although there is clearly a large degree of overlap. It is possible that single cell, or single nuclei, RNA-sequencing of human skeletal muscle could provide greater resolution to help clarify this issue.

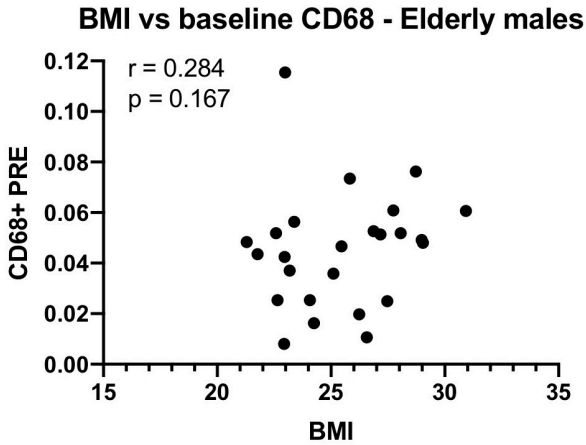
These findings indicate that categorizing macrophages into clearly defined pro- or anti-inflammatory subtypes may provide an inaccurate depiction of their complex functionality and how they contribute to remodeling in physiological settings. Though speculative, it is possible that the presence of pro- and anti-inflammatory markers indicates a transition from one subtype to another, or rather that most macrophages











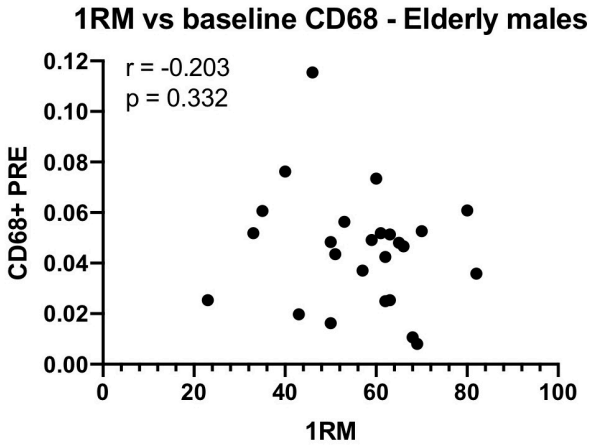


FIGURE 7 | Relationship between body mass index (BMI), or 1-repition maximum (1RM), and the number of CD68 macrophages counted on tissue sections from muscle biopsies obtained from young and elderly females, and elderly males. Spearman's *r* and the *p*-values for the correlation are displayed for each graph. Note that if the potential outlier in the CD68 data of the elderly males is removed, the *r* value is 0.373 and the *p*-value 0.073, suggesting a trend.

express many markers and rarely exist in pure pro- or anti-inflammatory states. It is likely that more severe models of injury induce more dramatic changes in the number of macrophages, where, given the sheer scale of tissue removal and rebuilding required, clearly distinct macrophage populations are observed (Saclier et al., 2013a). Indeed, some studies have shown that the range and duration of the presence of macrophages vary, based on the protocols used to induce muscle damage (Chazaud, 2014) raising the question regarding the role of the different macrophage subtypes present in situations of major regeneration versus tissue remodeling such as in the current study. In animals, significantly elevated concentrations of pro-inflammatory macrophages have been reported 24 h after muscle injury followed by an elevation of anti-inflammatory macrophages peaking 4–7 days post injury (Tidball and Villalta, 2010; Chazaud, 2015). Animal studies have also shown that disrupting the local transition from pro- to anti-inflammatory results in impaired or defective muscle regeneration (Saclier et al., 2013b). Another human study using eccentric muscle-damaging exercise found CD68+ cells peaking 4–7 days post exercise (Paulsen et al., 2012a) corresponding to the findings in our study. A study by Sorensen et al. (2019) investigated changes in local macrophage content following 300 lengthening contractions, a much more severe damaging model than ours, and found an increase in CD68+ cells 24H and 72H after exercise. It is likely that the reason we did not detect a significant increase at 24H post exercise is due to the lower exercise stimulus, or extent of damage, compared to that of Sorensen and colleagues. A study by Saclier et al. (2013a) in humans found that macrophage subtypes coexist in regenerative areas, where the prevalence of pro- and anti-inflammatory macrophages is determined by the phase of regeneration (early vs. late) in that specific area. In our male study we did not analyze for pro-inflammatory macrophages and therefore their presence remains unclear. Although speculative, the trend for alterations in TNF-a mRNA levels along with an immediate increase of a number of gene expression targets associated with pro-inflammatory macrophages (IL-1B, IL-1R) suggests an increased inflammatory environment prior to the increases we observed in CD163+ cells.

Regarding the resolution timeframe of tissue repair, we have previously detected elevated levels of CD68+ cells up to 30 days after using electrical stimulation to induce muscle necrosis (Mackey et al., 2011). At this time point the muscle is mostly repaired, but with clearly ongoing remodeling of the muscle connective tissue (Mackey et al., 2011, 2016) raising the possibility that macrophages exert anti-fibrotic actions, although this is speculative. Increases in COL1A1 gene expression were seen in the elderly men at 4 and 7 days post exercise. While the significance of these increased levels is difficult to interpret it indicates a heightened extracellular matrix remodeling following a heavy exercise bout. The timing of this regulation aligns with other findings from our laboratory demonstrating that ECM remodeling increases at later (>7 days) time points after exercise or injury (Mackey et al., 2016; Mackey and Kjaer, 2017; Karlsen et al., 2020). It is worth noting that 3 months after a muscle injury protocol and subsequent resistance training, COL1A1 mRNA was still upregulated in the muscle of elderly, but not in that of young, individuals (Karlsen et al., 2020) similar to the present study where greater COL1A1 mRNA levels were seen in the elderly vs. young females 5 days following the exercise bout. While the reason for this discrepancy between young and elderly is unclear it is possible that it simply represents a longer tissue healing trajectory for the older muscle.

Progressive impairment of skeletal muscle function with aging has been linked to disequilibrium between muscle damage and repair (Cui et al., 2019; Reidy et al., 2019). The findings in our study display some discrepancies between young and old subjects which may relate to this. In the elderly muscle, trends for higher content of macrophages were detected compared to the young group. While further research is necessary to fully understand the implication of these differences, it is worth noting the large spread in macrophage numbers and that we did observe a relationship between BMI and the number of macrophages (CD68+) in the control leg of the young females and a trend for a similar relationship in the elderly females (and elderly males with removal of a potential outlier). These individuals were recruited as generally healthy although there is likely to be a continuum from the higher BMI registered for some of our participants into the obese category. The high variation in macrophage content in human muscle biopsies has been reported by others, where it was found to be associated with walking performance in patients with peripheral artery disease (Kosmac et al., 2020). Similarly, the finding of an association between macrophages and muscle hypertrophy in human skeletal muscle (Walton et al., 2019b) clearly suggests a role for macrophages in the adaptive response to physiological exercise, as well as the clinical implications for macrophages in human skeletal muscle in the context of disease. Unfortunately, our study design was not suited to further explore these issues, so future work could investigate for example how the acute macrophage response to exercise is related to the hypertrophy response in the long term, and perhaps more pertinently, in the context of elderly populations.

The exercise induced increase seen for CD68+ cells and CD206+ cells in elderly females was expected and aligns with the findings observed among the male participants. Unexpectedly, no changes in macrophage counts were found in the young group, in contrast to other studies detecting increased numbers of pro-inflammatory macrophages in young individuals following a heavy resistance exercise bout (Przybyla et al., 2006; Sorensen et al., 2019). Whether our findings are related to age *per se* is difficult to determine. Indeed it is possible that muscle damage is the main driver of the macrophage response. However, we detected similar increases in circulating creatine kinase in the young and elderly females at 5 days post exercise, together with similar numbers of fibers positive for neonatal myosin at this time point (Bechshøft et al., 2019; Soendenbroe et al., 2020) suggesting that muscle damage leading to necrosis was absent. This is further supported

by the complete lack of fibers containing macrophages in the present study. While these findings indicate that no major damage was induced by our protocol, we cannot rule out the possibility that a lesser response in the young females is due to the exercise stimulus being less unaccustomed due to a generally more active lifestyle. However, we did not account for this in the current study and can therefore not conclude that our findings are due to age *per se*.

Together the findings of this study provide insight into the time course of macrophage activity and associated molecular targets in the context of aging and exercise. With most of the current understanding of macrophages in skeletal muscle originating from only a handful of human studies or animal studies utilizing severe damage protocols, our findings provide a time course for macrophage infiltration in conjunction with the molecular response to a physiological bout of exercise in elderly human skeletal muscle, adding to the understanding of the interaction between macrophages, inflammation and the aging muscle. Interestingly, we report that the most prevalent macrophage phenotype present in healthy human skeletal muscle expresses multiple markers, questioning the classification into clearly defined pro- and anti-inflammatory subtypes, at least in healthy muscle where necrosis is absent. Lastly, the relationship between the muscle macrophages content in the rested state and variables such as BMI and 1RM are in line with recent work (Kosmac et al., 2020) placing muscle macrophages in a clear clinical context with regard to physical function and disease. Extricating the specific roles of macrophages in aging as such from this complex matrix requires further study.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Committees on Health Research Ethics for the Capital Region of Denmark. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SJ, CB, MH, PS, JA, MK, and AM contributed to the conception and design of the study. SJ, MH, and CB recruited the participants and carried out the study. SJ performed the immunohistochemical, mRNA, and statistical analyses. SJ and AM wrote the first draft of the manuscript. All the authors contributed to manuscript revision, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.00811/full#supplementary-material

FIGURE S1 | Changes in muscle CD68+ cells **(top)** and CD68+CD163+ cells **(bottom)** in healthy elderly men receiving placebo (n = 13) or Losartan (n = 12) over a 7-day time course following an acute bout of heavy resistance leg extension exercise. Data were log-transformed and analyzed using a two-way repeated measures ANOVA (treatment × time) and Dunnett's *post hoc* test to compare for an effect of time compared with PRE. Data are shown as geometric mean ± back transformed SEM. *P < 0.05 time compared with PRE. Tendencies are written.

FIGURE S2 | Gene expression in the muscle biopsies of elderly men receiving placebo (n = 13) or Losartan (n = 12). mRNA data were normalized to RPLP0, log-transformed and are shown as geometric mean ± back transformed SEM, relative to baseline (−10 days). Data were analyzed with a two-way repeated measures ANOVA (treatment × time). *P < 0.05 compared with baseline. Tendencies are written.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Isoquercitrin Delays Denervated Soleus Muscle Atrophy by Inhibiting Oxidative Stress and Inflammation

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Although denervated muscle atrophy is common, the underlying molecular mechanism remains unelucidated. We have previously found that oxidative stress and inflammatory response may be early events that trigger denervated muscle atrophy. Isoquercitrin is a biologically active flavonoid with antioxidative and anti-inflammatory properties. The present study investigated the effect of isoquercitrin on denervated soleus muscle atrophy and its possible molecular mechanisms. We found that isoquercitrin was effective in alleviating soleus muscle mass loss following denervation in a dose-dependent manner. Isoquercitrin demonstrated the optimal protective effect at 20 mg/kg/d, which was the dose used in subsequent experiments. To further explore the protective effect of isoquercitrin on denervated soleus muscle atrophy, we analyzed muscle proteolysis *via* the ubiquitin-proteasome pathway, mitophagy, and muscle fiber type conversion. Isoquercitrin significantly inhibited the denervation-induced overexpression of two muscle-specific ubiquitin ligases—muscle RING finger 1 (MuRF1) and muscle atrophy F-box (MAFbx), and reduced the degradation of myosin heavy chains (MyHCs) in the target muscle. Following isoquercitrin treatment, mitochondrial vacuolation and autophagy were inhibited, as evidenced by reduced level of autophagy-related proteins (ATG7, BNIP3, LC3B, and PINK1); slow-to-fast fiber type conversion in the target muscle was delayed *via* triggering expression of peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α); and the production of reactive oxygen species (ROS) in the target muscle was reduced, which might be associated with the upregulation of antioxidant factors (SOD1, SOD2, NRF2, NQO1, and HO1) and the downregulation of ROS production-related factors (Nox2, Nox4, and DUOX1). Furthermore, isoquercitrin treatment reduced the levels of inflammatory factors—interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α)—in the target muscle and inactivated the JAK/STAT3 signaling pathway. Overall, isoquercitrin may alleviate soleus muscle atrophy and mitophagy and reverse the slow-to-fast fiber type conversion following denervation *via* inhibition of oxidative stress and inflammatory response. Our study findings enrich the knowledge regarding the molecular regulatory mechanisms of denervated muscle atrophy and provide a scientific basis for isoquercitrin as a protective drug for the prevention and treatment of denervated muscle atrophy.

Keywords: denervated muscle atrophy, isoquercitrin, oxidative stress, inflammation, proteolysis, mitophagy

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INTRODUCTION

Skeletal muscle, an integral part of the human body, is involved in various bodily functions including exercise, assisted breathing, heat production, internal organ protection, and glucose and fat metabolism. Skeletal muscle comprises approximately 40% of the total body weight. Maintenance of skeletal muscle homeostasis is essential for maintaining the body's various functions (Ferreira et al., 2019; Yamakawa et al., 2020). An imbalance in skeletal muscle homeostasis can cause skeletal muscle hyperplasia or atrophy. Skeletal muscle atrophy is characterized by reduced muscle mass and muscle fibers, weakened muscle strength, and functional decline. Patients with severe muscle atrophy can lose the ability to move and are bedridden. Long-term bedridden conditions aggravate muscle atrophy and cause various life-threatening complications (Ceco et al., 2017; Sakellariou and McDonagh, 2018). Skeletal muscle atrophy commonly occurs concomitant to various conditions, including peripheral nerve injury, weightlessness, limb immobilization, aging, and numerous diseases (e.g., cancer cachexia, diabetes, heart failure, and kidney failure; Hyatt et al., 2019; Salucci and Falcieri, 2020).

Peripheral nerve injury is a common disease that inevitably causes a certain degree of neuronal degeneration, muscle atrophy, and fibrosis. During the repair of long-distance nerve defects, target muscle atrophy is often irreversible because the regenerated nerve grows gradually and is unable to reach the target muscle in time. Consequently, muscle atrophy critically affects the functional reconstruction of the target muscle, placing a substantial burden on the patient's family and on society (Tuffaha et al., 2016). Therefore, promoting nerve regeneration ability and delaying skeletal muscle atrophy progression are crucial for improving the functional reconstruction of the injured nerve (Tos et al., 2013; Chiono and Tonda-Turo, 2015). Although a substantial amount of research on denervated muscle atrophy has been conducted, a sound method for the prevention and treatment of denervated muscle atrophy is lacking, which may be owing to a poor understanding of the molecular mechanisms involved.

Muscle atrophy is characterized by a decreased contractility of muscle fibers, changes in muscle fiber types and myosin subtypes, and a net loss of cytoplasm, organelles, and total proteins (Dumitru et al., 2018). In recent years, although research on denervated muscle atrophy has made substantial progress, it has mainly focused on single events, genes, or proteins (Li et al., 2017; Reza et al., 2017; Gueugneau et al., 2018; Yin et al., 2018; Castets et al., 2019; Janice Sánchez et al., 2019). Because systematic research on denervated muscle atrophy has not yet been conducted, no breakthrough has been achieved. Numerous molecules involved in denervated muscle atrophy are also involved in several events or pathways that are interconnected, including proteolytic pathways (such as the ubiquitin-proteasome pathway and autophagy-lysosomal pathway), protein synthesis pathways, and muscle fiber regeneration pathways (Winbanks et al., 2016; Arouche-Delaperche et al., 2017; Li et al., 2017; Quattrocelli et al., 2017; Brzeszczyńska et al., 2018; Yin et al., 2018; Huang et al., 2019; Qiu et al., 2019; Wu et al., 2019). Moreover, interconnections between these pathways complicate the molecular mechanism of denervated muscle atrophy. Therefore, it is crucial to identify targets that can effectively delay the process of muscle atrophy, emphasizing on the search for an upstream factor or event that initially triggers muscle atrophy, to provide new strategies for the prevention and treatment of denervated muscle atrophy.

Considering this scenario, our research group has performed a series of studies on denervated muscle atrophy using genomics and proteomics. We have found that proteolysis *via* the ubiquitin-proteasome and autophagy-lysosomal proteolytic athways and the protein synthesis pathway plays an important role in the process of denervated muscle atrophy (Sun et al., 2006, 2009, 2012, 2014a,b; He et al., 2016; Qiu et al., 2018). Recently, we used transcriptome sequencing and bioinformatics methods to systematically analyze the differentially expressed genes involved in denervated muscle atrophy. To the best of our knowledge, it was proposed for the first time that denervated muscle atrophy can be divided into four different transcription stages (Shen et al., 2019). In the oxidative stress stage (0–12 h), oxidative stress occurs early after skeletal muscle denervation, which may be attributed to the loss of contractile function of the target muscle, resulting in decreased blood perfusion in the muscle. Therefore, under a relatively hypoxic state, reactive oxygen species (ROS) will be produced. Persistent hypoxia results in excessive ROS production, causing an imbalance between the oxidation and antioxidation systems. In the inflammation stage (24 h), excessive ROS production causes tissue damage, thereby inducing the generation of numerous inflammatory factors. These inflammatory factors cause further inflammation by activating inflammatory response pathways. In the atrophic (3–7 days) and atrophic fibrosis (14–28 days) stages, excessively activated inflammation further initiates the downstream muscle atrophic process, thereby promoting target muscle atrophy and fibrosis (Shen et al., 2019). Overall, oxidative stress, inflammation, atrophy, and atrophic fibrosis sequentially occur following skeletal muscle denervation. Previous studies have reported that high ROS levels can cause proteolysis, muscle cell apoptosis, and eventual skeletal muscle atrophy (Theilen et al., 2017; Ábrigo et al., 2018; Guigni et al., 2018). Muller et al. (2007) found a significant increase in ROS levels in the aging and skeletal muscles of patients with amyotrophic lateral sclerosis, and the ROS level was closely related to the degree of skeletal muscle atrophy. Other studies have indicated that inflammation is involved in skeletal muscle atrophy caused by tumor cachexia and sepsis (Zhu et al., 2017; Cerquone Perpetuini et al., 2018). These results suggest that oxidative stress and inflammatory signals that are sequentially activated within 24 h following skeletal muscle denervation are essential for triggering denervated skeletal muscle atrophy.

Isoquercitrin (quercetin-3-O-β-D-glucopyranoside) is a flavonoid compound widely distributed in plants. It possesses various biological properties, such as anti-inflammatory, antioxidative, anti-allergic, and anti-viral activities (Li et al., 2019).

Abbreviations: DHE, Dihydroethidium; ELISA, Enzyme-linked immunosorbent assay; ICR, Institute of Cancer Research; MAPK, Mitogen-activated protein kinase; MyHC, Myosin heavy chain; PBS, Phosphate-buffered saline; PCR, Polymerase chain reaction; PVDF, Polyvinylidene difluoride; ROS, Reactive oxygen species; TBST, Tris-buffered saline with Tween; TEM, Transmission electron microscopy.

Existing studies have found that isoquercitrin exerts a neuroprotective effect on ischemic stroke. By activating the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway, isoquercitrin promotes the expression of antioxidant enzymes, thereby inhibiting the NOX4/ROS/nuclear factor κB (NF-κB) pathway and reducing oxidative stress and neuronal apoptosis (Dai et al., 2018). Reportedly, isoquercitrin improves the production of inflammatory factors, including tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6, by blocking the NF-κB and mitogen-activated protein kinase (MAPK) pathways for protecting the liver from acetaminophen-induced damage (Xie et al., 2016), reduces high-fat diet- and beta-amyloid-induced oxidative stress for improving cognitive function in mice (Kim et al., 2019), and upregulates Nrf2 expression, inhibits the NF-kB pathway, and regulates the AMP-activated protein kinase pathway to alleviate streptozotocin-induced diabetic symptoms in rats (Jayachandran et al., 2019). However, studies on whether isoquercitrin can alleviate muscle atrophy and the underlying molecular mechanisms are lacking.

Therefore, in the present study, we established a denervated muscle atrophy model using sciatic nerve disruption in mice, followed by intraperitoneal injection. The atrophy of soleus muscle, containing 98% slow type muscle fibers, was greater affected by sciatic nerve transection as compared to tibialis anterior and extensor digitorum longus muscles (Beehler et al., 2006; Higashino et al., 2013). The wet weight ratio, muscle fiber cross-sectional area (CSA), mitophagy, and muscle fiber type conversion of the soleus muscle were investigated to evaluate the protective effect of isoquercitrin on denervated muscle atrophy. Expression of genes and proteins related to inflammation and oxidative stress was determined to analyze the possible mechanism *via* which isoquercitrin delays denervated muscle atrophy. This study attempted to further enrich the knowledge regarding the molecular mechanism of denervated muscle atrophy and to provide a scientific basis for isoquercitrin as a protective drug for the prevention and treatment of muscle atrophy.

MATERIALS AND METHODS

Animal Experiment

Healthy (6–8 weeks old) male Institute of Cancer Research (ICR) mice (weight, 20 ± 2 g) were provided by the Experimental Animal Center of Nantong University, China. The experiments involving animals were carried out in accordance with the animal care guidelines of Nantong University and ethically approved by Jiangsu Administration Committee of Experimental Animals. The mice were anesthetized using an intraperitoneal injection of a compound anesthetic, and the sciatic nerve was exposed and further isolated. A 1-cm sciatic nerve transection was made in the femoral segment of the left hind limb, which was then disinfected and sutured. The mice were randomized into the following groups: sham operation group (Ctrl), denervation group (DEN), and denervation + isoquercitrin (10, 20, and 40 mg/kg/d) group (ISO-L, ISO-M, and ISO-H). Isoquercitrin or saline was administrated by intraperitoneal injection after sciatic nerve transection and lasted for 14 consecutive days in each group, respectively. Thereafter, the mice were killed, and the bilateral soleus muscles of the hind limbs of each mouse were collected, weighed, and stored using different storage methods as per the requirements of subsequent experiments. The wet weight ratio of muscle was calculated by the injury side compared with the contralateral side.

Immunofluorescence Staining

Following sample fixation with 4% paraformaldehyde, the entire soleus muscle was removed and dehydrated in a series of 10, 20, and 30% sucrose. Thereafter, one-third of the muscles were embedded in optimal cutting temperature (OCT) compound, frozen, and sliced into 8-μm-thick sections. These sections were mounted and incubated overnight with primary antibodies for laminin or fast myosin skeletal heavy chain (Abcam, Cambridge, UK). On the following day, the sections were rinsed with phosphate-buffered saline (PBS), incubated with the corresponding secondary antibodies at room temperature for 1–2 h, rinsed with PBS, mounted, dried, and photographed using a Zeiss fluorescent microscope.

Dihydroethidium Probe for Determination of ROS Level

The mice were irrigated with saline at room temperature, followed by perfusion with dihydroethidium (DHE) solution (10 μM, Beyotime, Haimen, China) for 1 h. Thereafter, they were perfused with 4% paraformaldehyde, following which the soleus muscles were removed and dehydrated in a series of 10, 20, and 30% sucrose. One-third of the soleus muscles obtained was embedded in OCT compound, and 8-μm-thick frozen sections were prepared and directly observed using the Zeiss fluorescent microscope.

Quantitative Reverse Transcription-Polymerase Chain Reaction

Using quantitative reverse transcription–polymerase chain reaction (qRT-PCR), levels in messenger RNAs (mRNAs) were analyzed, as described (Huang et al., 2019). In short, total RNA was extracted from soleus muscle samples and used to generate cDNA samples. PCR was performed according to our previous study. The primers were as follows: mouse *Nrf2* Forward: TAGATGACCATGAGTCGCTTGC, Reverse: GCCA AACTTGCTCCATGTCC; mouse *NQO1* Forward: AGGAT GGGAGGTACTCGAATC, Reverse: TGCTAGAGATGACT CGGAAGG; mouse *HO-1* Forward: AGGTACACATCCAAG CCGAGA, Reverse: CATCACCAGCTTAAAGCCTTCT; mouse *IL-6* Forward: CTGCAAGAGACTTCCATCCAG, Reverse: AGT GGTATAGACAGGTCTGTTGG, mouse *IL-1β* Forward: GAAA TGCCACCTTTTGACAGTG, Reverse: TGGATGCTCTCATC AGGACAG; mouse *TNF-α* Forward: CAGGCGGTGCCTAT GTCTC, Reverse: CGATCACCCCGAAGTTCAGTAG; mouse *SOD1* Forward: AACCAGTTGTGTTGTCAGGAC, Reverse: CCACCATGTTTCTTAGAGTGAGG; mouse *SOD2* Forward: CAGACCTGCCTTACGACTATGG, Reverse: CTCGGTGGCGTT GAGATTGTT; mouse *Duox1* Forward: TATCTCCCCAGAG TTCGTTGT, Reverse: GGGTGCTCTCGACTCCAGT; mouse *GAPDH* Forward: AACTTTGGCATTGTGGAAGG, Reverse:

ACACATTGGGGGTAGGAACA; The relative mRNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) level was used as the internal control.

Western Blot

The total protein in the muscle was extracted from the protein lysate. Protein concentration was measured using a BCA kit (Beyotime, Haimen, China). Thereafter, 30 μg of the total protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane *via* the wet transfer method. The PVDF membrane was blocked for 1 h, followed by incubation with the primary antibody antibodies: mouse anti-MyHC (R&D Systems, Minneapolis, MN), rabbit anti-NOX4 (Invitrogen, Rockford, IL, USA), rabbit anti-MAFbx, rabbit anti-PINK1, rabbit anti-ATG7, mouse anti-BNIP3, rabbit anti-LC3B, rabbit anti-NOX2, rabbit anti-Nrf2, rabbit anti-NQO1, rabbit anti-PGC-1α (Abcam, Cambridge, UK), mouse anti-p-Jak1 (Tyr1034/1035)/Jak2 (Tyr1007/1008), rabbit anti-p-Stat3 (Tyr705) and rabbit anti-Stat3 (Cell Signaling technology, MA, USA), rabbit anti-MuRF-1, mouse anti-Troponin I-FS, mouse anti-Troponin I-SS, and mouse anti-tubulin (Santa Cruz, Santa Cruz, CA) at 4°C overnight. On the following day, the PVDF membrane was rinsed thrice with tris-buffered saline with Tween (TBST) and incubated with the corresponding secondary antibody at room temperature for 1 h. Thereafter, PVDF membrane was rinsed thrice with TBST, treated with the appropriate amount of luminescent liquid, and finally scanned using a membrane scanner.

Transmission Electron Microscopy Analysis

To observe the changes in the mitochondria, the soleus muscle was analyzed through transmission electron microscopy (TEM) analysis, as previously reported (Huang et al., 2019). Briefly, 1-mm^3-sized muscle was fixed in 2.5% glutaraldehyde, followed by post fixation in 1% osmium tetroxide. Muscle sections (20 fields per mouse and three mouse per group) were analyzed by TEM (HT7700, Hitachi, Tokyo, Japan). The number of vacuoles or autophagosomes per 100 mitochondria was calculated.

Enzyme-Linked Immunosorbent Assay

To observe the changes of proinflammatory cytokines, the content of IL-1β, IL-6, and TNF-α was measured according to the manufacturer's instructions. Briefly, enzyme-linked immunosorbent assay (ELISA) plates (Beyotime, Haimen, China) were incubated with 100 μl muscle lysates at 37°C for 2 h, followed by incubation with anti-IL-1β, anti-IL-6, or anti-TNF-α antibodies for 1 h. Subsequently, ELISA plates were washed and incubated with horseradish peroxidase (HRP)-streptavidin for 20 min. Absorbance (450 nm) was measured using a microplate spectrophotometer.

Statistical Analysis

Data in this study were analyzed using one-way ANOVA, followed by the Tukey's multiple comparisons test. All statistical analyses were conducted with GraphPad Prism software (version 7.0; San Diego, CA, USA). The level of significance was set at $p < 0.05$.

RESULTS

Isoquercitrin Relieves Skeletal Muscle Atrophy Caused by Denervation

We used ICR adult mice to prepare sciatic nerve transection models. The mice were randomly divided into sham operation, DEN, low-dose isoquercitrin (10 mg/kg/d), middle-dose isoquercitrin (20 mg/kg/d), and high-dose isoquercitrin (40 mg/kg/d) groups. The soleus muscle of each mouse was obtained after 2 weeks of treatment, and the wet weight ratio of the soleus muscle was analyzed. The target muscle wet weight ratio in the DEN was significantly lower than that in the control group ($p < 0.001$), indicating that the denervated muscle atrophy model was successfully prepared. The target muscle wet weight ratios in the isoquercitrin-treated groups were significantly higher than that in DEN, indicating that isoquercitrin alleviates skeletal muscle atrophy caused by denervation in a dose-dependent manner. Middle-dose isoquercitrin (20 mg/kg/d) demonstrated the optimal protective effect in the mouse model of denervated muscle atrophy (**Figure 1**).

Laminin immunofluorescence staining performed to further investigate the effect of isoquercitrin on denervated muscle atrophy revealed that the muscle fiber cross-sectional area in DEN was significantly smaller than that in the control group, indicating that denervation significantly reduced the muscle fiber cross-sectional area. Compared with DEN, the muscle fiber cross-sectional area in the low-dose isoquercitrin group was larger but not of significance, whereas the muscle fiber cross-sectional area in the middle- and high-dose isoquercitrin group was significantly larger ($p < 0.01$). It was suggested that isoquercitrin inhibits the reduction in the muscle fiber cross-sectional area caused by denervation, and the middle-dose isoquercitrin showed the optimal protective effect (**Figure 1**). These findings were consistent with the results of the target muscle wet weight ratio, indicating that isoquercitrin intervention can effectively alleviate denervated muscle atrophy.

Isoquercitrin Inhibits Proteolysis *via* the Ubiquitin-Proteasome Pathway

Because middle-dose isoquercitrin demonstrated the best effect for preventing and treating denervated muscle atrophy, it was used in subsequent experiments. We measured the expression of the ubiquitin-proteasome proteolytic system during denervated muscle atrophy to further explore the possible mechanism of isoquercitrin in alleviating this condition. The ubiquitin-proteasome system plays an important role in various muscle atrophies (Winbanks et al., 2016; Quattrocelli et al., 2017; Qiu et al., 2019). Our study found that the expression of the muscle-specific ubiquitin ligases muscle atrophy F-box (MAFbx) and muscle RING finger 1 (MuRF1) in DEN was significantly higher than that in the control group. Therefore, the ubiquitin-proteasome proteolytic system in the target muscle was significantly activated following denervation, thereby enhancing the protein degradation ability. In contrast, isoquercitrin treatment significantly reduced the expression of MAFbx and MuRF1 in the denervated muscle and inhibited the activation of the ubiquitin-proteasome proteolytic

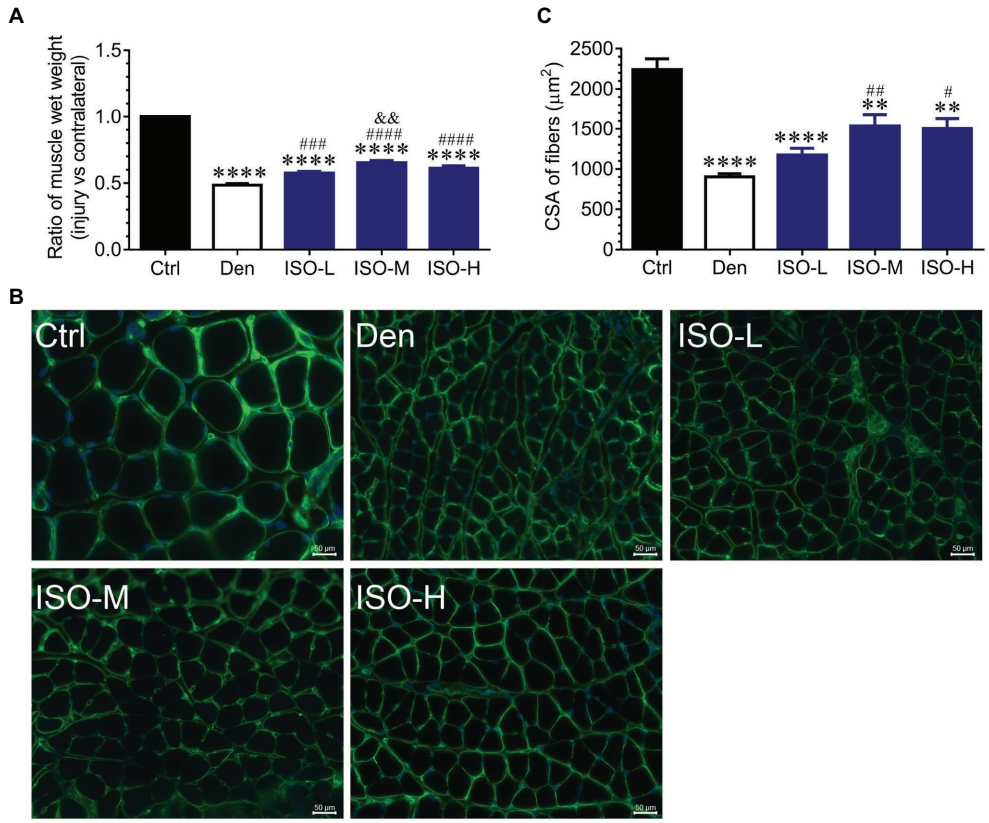

FIGURE 1 | Isoquercitrin reduces soleus muscle mass loss caused by denervation (n = 6). **(A)** The ratio of muscles wet weight in each group. **(B)** Representative images of laminin-stained muscles cross-sections in each group. Green indicates laminin staining. Scale bar: 50 μm. **(C)** The histogram shown the cross-sectional area (CSA) of muscles in each group. Ctrl, control group; Den, denervation group; ISO-L, denervated target muscle plus low-dose isoquercitrin (10 mg/kg/d); ISO-M, denervated target muscle plus middle-dose isoquercitrin (20 mg/kg/d); ISO-H, denervated target muscle plus high-dose isoquercitrin (40 mg/kg/d). $^{**}p < 0.01$ and $^{****}p < 0.0001$ vs. Ctrl; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, and $^{\#\#\#\#}p < 0.0001$ vs. Den; $^{\&\&}p < 0.01$ vs. ISO-L.

system in the target muscle following denervation, thereby reducing the proteolytic capacity. Myosin is the main contractile and regulatory protein in skeletal muscle fibers, and myosin heavy chain (MyHC), an important component of myosin, can be used to determine the extent of muscle protein degradation (Tajsharghi and Oldfors, 2013). In the present study, the expression of MyHC in DEN was significantly lower than that in the control group, whereas its expression in the isoquercitrin-treated groups was significantly higher than that in DEN (**Figure 2**), which may be attributed to the changes in the expression of MAFbx and MuRF1. These findings indicated that isoquercitrin can delay denervated skeletal muscle atrophy progression by inhibiting proteolysis *via* the ubiquitin-proteasome pathway and by reducing myosin degradation.

Isoquercitrin Reduces Mitophagy Caused by Skeletal Muscle Denervation

Studies have shown that autophagy plays an important role in the muscle atrophic process (Milan et al., 2015). In our study, the number of vacuoles in the DEN was significantly higher than that in the control group, which indicated that vacuolar degeneration and autophagy were observed in a large number of mitochondria following target muscle denervation, accompanied by the high expression of the autophagy-related proteins ATG7, BNIP3, PINK1, and LC3B (**Figure 3**). However, isoquercitrin treatment significantly inhibited mitochondrial vacuolar degeneration and autophagy and downregulated the expression of ATG7, BNIP3, PINK1, and LC3B (**Figure 3**). These findings suggested that isoquercitrin alleviates mitophagy by downregulating the expression of autophagy-related proteins in denervated target muscles, thereby delaying denervated muscle atrophy.

Isoquercitrin Delays the Slow-to-Fast Fiber Type Conversion Caused by Denervation

The conversion between muscle fiber types is an important feature of muscle atrophy. Studies have reported that denervation-induced slow-to-fast fiber type conversion severely impacts the normal function of the target muscle. Immunofluorescence staining of fast muscle fiber protein indicated that the proportion of fast muscle fibers in the soleus muscle following denervation was significantly higher than that in the control group. Moreover, western blots revealed that the expression of fast skeletal muscle troponin I (TnI-FS) significantly increased and that of slow skeletal

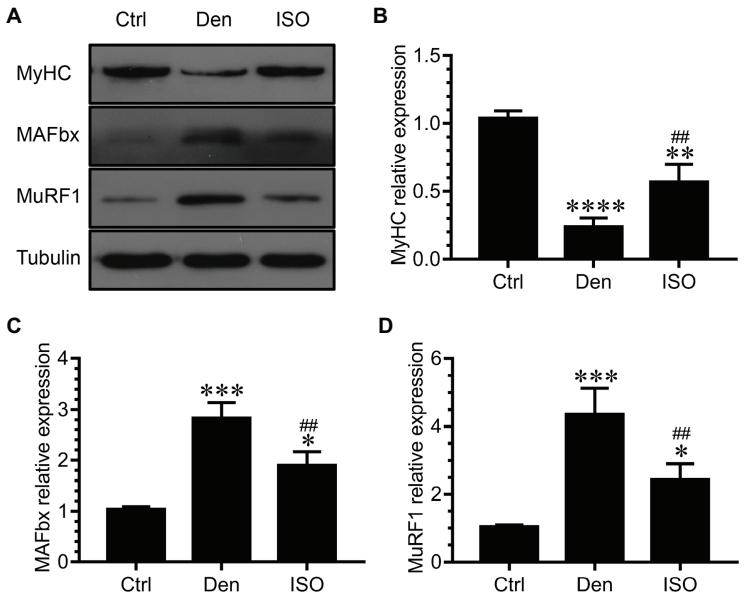

FIGURE 2 | Isoquercitrin inhibits proteolysis *via* the ubiquitin-proteasome pathway ($n = 6$). **(A)** Western blot of MyHC, MAFbx, and MuRF1. **(B–D)** Quantification of the expression of MyHC, MAFbx, and MuRF1. Ctrl, control group; Den, denervation group; ISO, denervated target muscle plus middle-dose isoquercitrin (20 mg/kg/d) group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ vs. Ctrl; $^{\#\#}p < 0.01$vs. Den. MyHC, myosin heavy chain; MuRF1, muscle RING finger 1; MAFbx, muscle atrophy F-box.

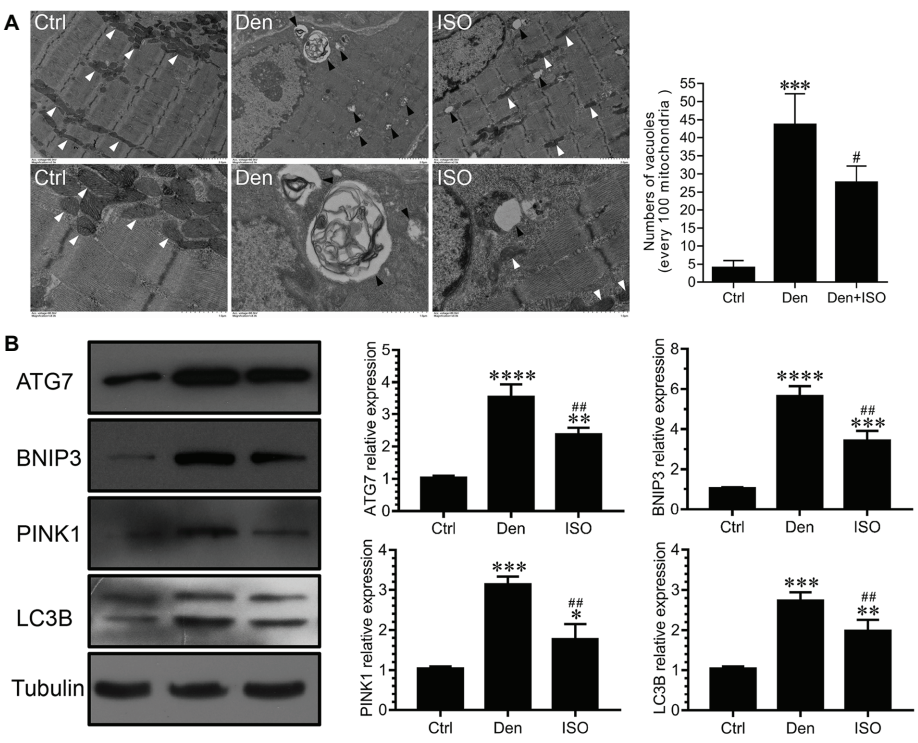

FIGURE 3 | Isoquercitrin reduces mitochondrial autophagy in denervated soleus muscles. **(A)** Ultrastructure of muscle fibers observed using transmission electron microscopy ($n = 3$). The white arrow indicates mitochondria between muscle fibers. The black arrow indicates an autophage or an autophagic vesicle. **(B)** Western blot and quantification of the autophagy-related proteins ATG7, BNIP3, PINK1, and LC3B ($n = 6$). Ctrl, control group; Den, denervation group; ISO, denervated target muscle plus isoquercitrin (20 mg/kg/d) group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ vs. Ctrl; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. Den.

muscle troponin I (TnI-SS) significantly decreased in the soleus muscle following denervation, suggesting the conversion from slow to fast muscle fibers following denervation. After isoquercitrin intervention, the proportion of fast muscle fibers in the denervated target muscle was significantly decreased, accompanied by TnI-FS downregulation and TnI-SS upregulation. Therefore, isoquercitrin could delay the slow-to-fast fiber type conversion caused by denervation. Peroxisome proliferator-activated receptor γ coactivator 1-alpha (PGC-1α) is a key factor that regulates mitochondrial function, regulates muscle fiber types, and controls the fast-to-slow muscle fiber type conversion (Lin et al., 2002). In the present study, isoquercitrin treatment significantly inhibited a reduction in the expression of PGC-1α in denervated target muscles (**Figure 4**). These results suggested that isoquercitrin reverses the slow-to-fast fiber type conversion by promoting the expression of PGC-1α, thereby protecting denervated muscle atrophy.

Isoquercitrin Inhibits the Inflammatory Response Caused by Target Muscle Denervation

Inflammation is involved in skeletal muscle atrophy caused by tumor cachexia and sepsis, and inflammation inhibition can alleviate muscle atrophy (Zhu et al., 2017; Cerquone Perpetuini et al., 2018). However, it is unclear whether isoquercitrin activity can delay denervated muscle atrophy by suppressing inflammation. Using qRT-PCR and ELISA, we observed that the expression of inflammation-related genes and proteins (IL-1β, IL-6, and TNF-α) in denervated target muscles was significantly increased, suggesting that these inflammatory factors are involved in denervated muscle atrophy. Isoquercitrin treatment significantly inhibited the elevation of the inflammatory factors IL-1β, IL-6, and TNF-α in denervated target muscles (**Figures 5A,B**). JAK/STAT3 is a classic signaling pathway downstream of IL-6. We observed that although pJAK2, pSTAT3, and STAT3 were significantly overexpressed in denervated target muscles, isoquercitrin could significantly inactivate the JAK2/STAT3 signals (**Figure 5C**). These results revealed that isoquercitrin may alleviate denervated muscle atrophy by relieving inflammation.

Isoquercitrin Inhibits Oxidative Stress in Denervated Target Muscles

Studies have reported that high ROS levels can cause proteolysis, muscle cell apoptosis, and eventual skeletal muscle atrophy (Theilen et al., 2017; Ábrigo et al., 2018; Guigni et al., 2018). Our previous study has suggested that excessive ROS causes oxidative stress damage, subsequently inducing the production of numerous inflammatory factors that cause inflammation (Shen et al., 2019). In the present study, we explored whether isoquercitrin can suppress the inflammatory response by suppressing oxidative stress. We used DHE probe detection to demonstrate that the

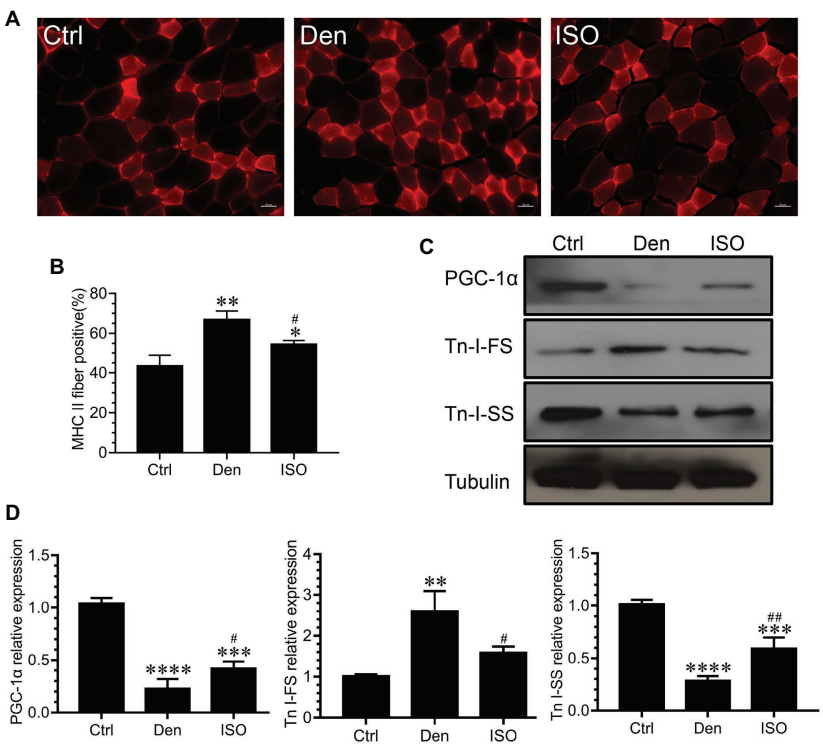

FIGURE 4 | Isoquercitrin delays the slow-to-fast fiber type conversion caused by denervation. **(A)** Immunofluorescence staining of fast myosin skeletal heavy chain in soleus muscle. **(B)** Quantification of the positive proption of fast muscle fibers ($n = 3$). **(C)** Western blot of PGC-1α, Tn I-FS, and Tn I-SS related to the slow-to-fast fiber type conversion. **(D)** Quantification of PGC-1α, Tn I-FS, and Tn I-SS. Ctrl, control group; Den, denervation group; ISO, denervated target muscle plus isoquercitrin (20 mg/kg/d) group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ vs. Ctrl; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. Den. PGC-1α, peroxisome proliferator-activated receptor γ coactivator 1α; TnI-FS, fast skeletal muscle troponin I; TnI-SS, slow skeletal muscle troponin I.

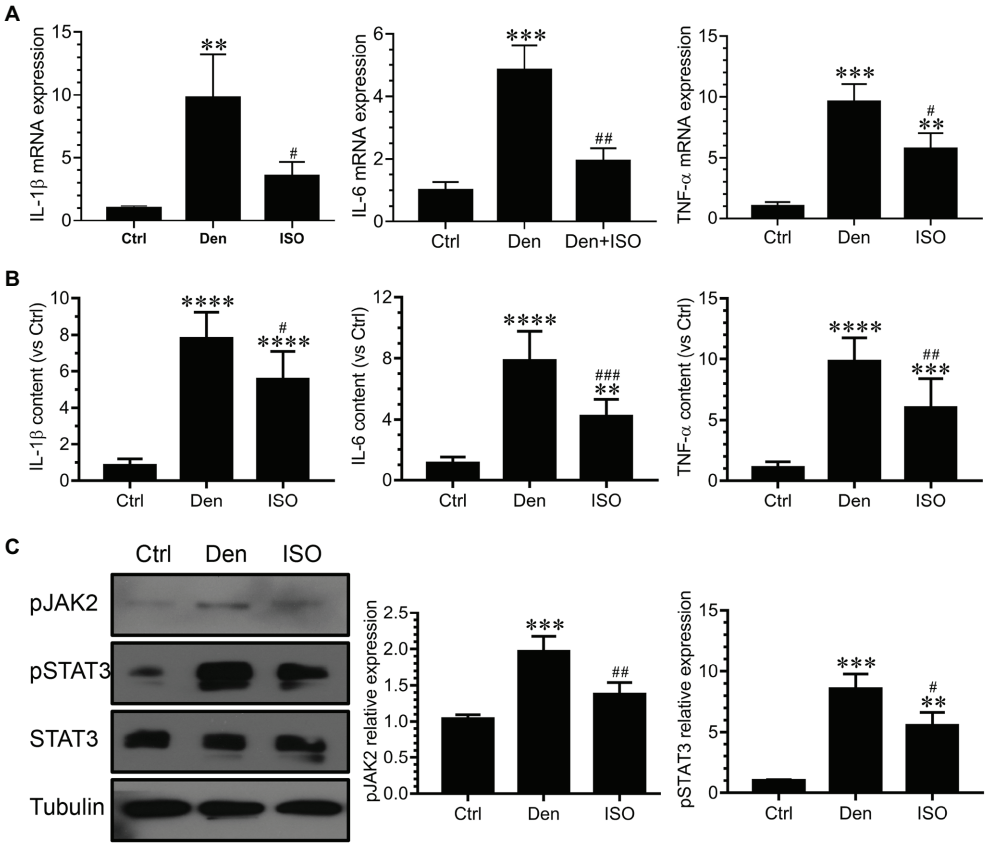

FIGURE 5 | Isoquercitrin inhibits the inflammatory response in denervated soleus muscles (n = 6). **(A)** Quantitative polymerase chain reaction detection of changes in the expression of the inflammatory factors interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α. **(B)** Enzyme-linked immunosorbent assay (ELISA) detection of the expression of IL-1β, IL-6, and TNF-α; **(C)** Western blot analysis of JAK/STAT3 activation. Ctrl, control group; Den, denervation group; ISO, denervated target muscle plus isoquercitrin (20 mg/kg/d) group. $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ vs. Ctrl; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ vs. Den.

ROS level in denervated target muscles was significantly higher than that in the control group and that isoquercitrin intervention could significantly inhibit the increase of ROS level in denervated target muscles (**Figures 6A,B**). qPCR and western blot findings showed that the expression of antioxidant-related genes and proteins (SOD1, SOD2, NRF2, NQO1, and HO1) in denervated target muscles significantly decreased and that of genes related to ROS production (Nox2, Nox4, and DUOX1) in denervated target muscles significantly increased. Isoquercitrin treatment significantly reversed the expression of antioxidant-related genes and proteins (SOD1, SOD2, NRF2, NQO1, and HO1) and ROS production-related genes (Nox2, Nox4, and DUOX1) in denervated target muscles (**Figure 6C**). These results suggested that isoquercitrin relieves denervated muscle atrophy by inhibiting oxidative stress and reducing inflammation.

DISCUSSION

Denervated muscle atrophy is a highly prevalent disease in clinical settings. Although several studies have been performed on denervated muscle atrophy and substantial progress has been made, there is no sound method for its prevention and treatment. The molecular mechanism of denervated muscle atrophy remains unelucidated (Cao et al., 2018). Currently, drug therapy for muscular atrophy mainly focuses on inhibiting protein degradation and promoting protein synthesis (Dutt et al., 2015). However, during the muscle atrophic process, there are several pathways involved in proteolysis and protein synthesis, such as the ubiquitin-proteasome system, autophagy-lysosome system, cathepsin hydrolysis, and insulin-like growth factor 1/phosphatidylinositide 3-kinase/protein kinase B-mediated anabolic pathways (Ma et al., 2019; Han et al., 2020; Nguyen et al., 2020). It is impractical to sequentially intervene in these pathways. If we can identify the early upstream trigger factors that concurrently regulate these pathways, interventions in these pathways may achieve better results.

Our previous study has shown that denervated muscle atrophy can be divided into oxidative stress, inflammatory, atrophic, and atrophic fibrosis stages. Because oxidative stress and inflammation can be sequentially activated within 24 h following skeletal muscle denervation, oxidative stress and inflammatory signals may play an essential early triggering role in denervated skeletal muscle atrophy (Shen et al., 2019). Therefore, the present study explored whether isoquercitrin, which has antioxidative and

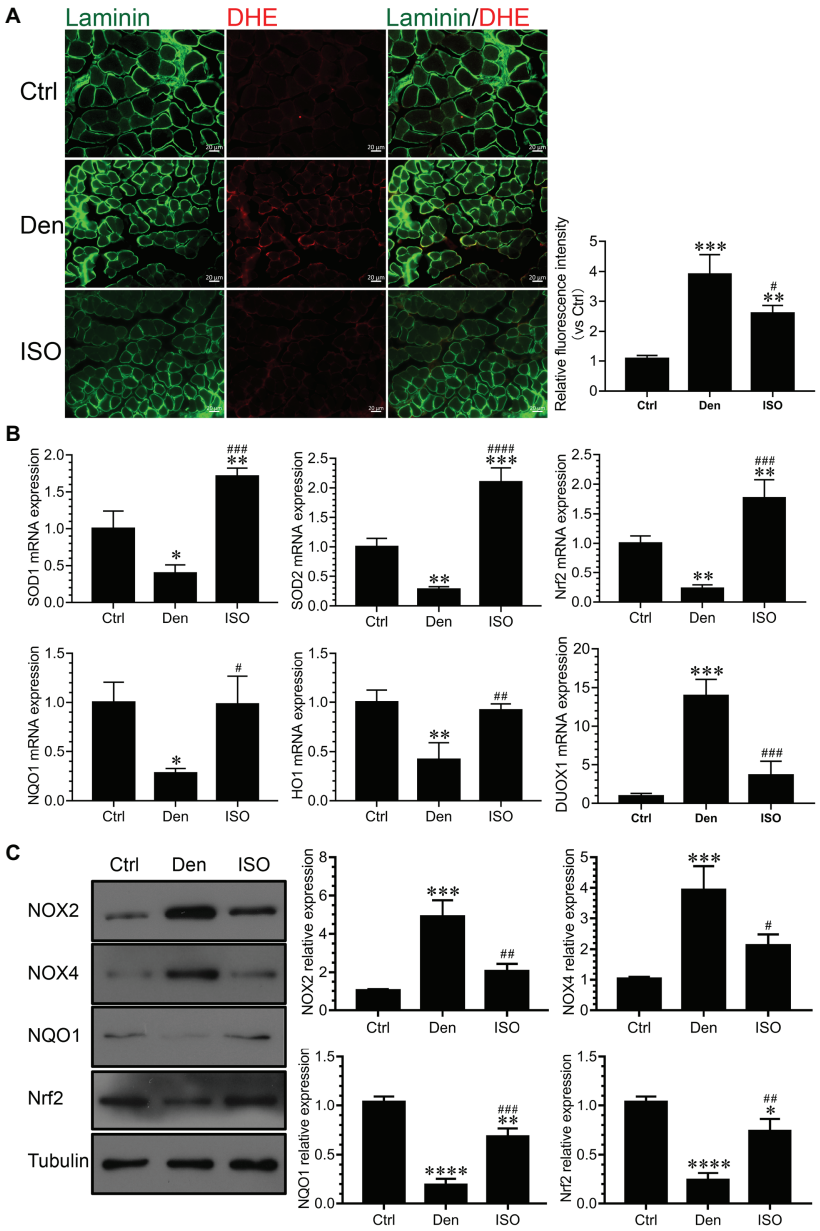

FIGURE 6 | Isoquercitrin inhibits oxidative stress in denervated soleus muscles (n = 6). **(A)** Fluorescence diagram of reactive oxygen species (ROS) level and quantification of relative fluorescence intensity of each group in the dihydroethidium (DHE) probe detection experiment. **(B)** Quantitative polymerase chain reaction detection of the relative expression of antioxidant-related genes and ROS production-related genes. **(C)** Western blot of ROS-related and antioxidant-related proteins and quantification of the relative expressions. ROS, reactive oxygen species; Ctrl, control group; Den, denervation group; ISO, denervated target muscle plus isoquercitrin (20 mg/kg/d) group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ vs. Ctrl; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, and $^{\#\#\#\#}p < 0.0001$ vs. Den.

anti-inflammatory activities, can alleviate denervated muscle atrophy. Our findings confirm the role of oxidative stress and inflammation in denervated muscle atrophy and provide a scientific basis for isoquercitrin as a protective drug for the prevention and treatment of muscle atrophy. Furthermore, our study revealed that isoquercitrin inhibits target muscle atrophy following denervation, which is mainly characterized as inhibiting the reduction of the wet weight ratio and muscle fiber cross-sectional area of the denervated target muscle. The ubiquitin-proteasome system plays an important role in various muscle atrophies, including those associated with denervation, disuse, tumor cachexia, diabetes, and various chronic inflammatory conditions (Gao et al., 2018; Lala-Tabbert et al., 2019; Han et al., 2020; Nguyen et al., 2020). MAFbx and MuRF1 are two muscle-specific E3 ubiquitin ligases that are increased in various muscle atrophies and are confirmed as suitable markers of muscle atrophy (Bodine and Baehr, 2014). In the present study, isoquercitrin inhibited the increase in the expression of MAFbx and MuRF1 in denervated

target muscles and reduced the degradation of MyHC, thereby alleviating denervated muscle atrophy. These results suggest that isoquercitrin relieves denervated muscle atrophy by inhibiting the ubiquitin-proteasome proteolytic system. Bandyopadhaya et al. (2019) reported that ROS accumulation may trigger the activity of the ubiquitin ligases MuRF1 and MAFbx. The expression of MAFbx and MuRF1 was inhibited by increase in the activity of the antioxidant factor HO1 in sepsis-induced muscle wasting (Yu et al., 2018a). Inflammation can induce muscle atrophy by enhancing the expression of MAFbx and MuRF1 (Hahn et al., 2020; Kim et al., 2020). TNF-α stimulates the expression of MAFbx in the skeletal muscle *via* the p38 MAPK pathway and promotes muscle fiber proteolysis (Ma, 2010). We observed that isoquercitrin can upregulate the expression of antioxidant factors (SOD1, SOD2, NRF2, NQO1, and HO1), downregulate the expression of ROS production-related factors (Nox2, Nox4, and DUOX1), inhibit the production of IL-1β, IL-6, and TNF-α, and inactivate JAK/STAT3 signaling. Therefore, isoquercitrin may reduce the expression of MAFbx and MuRF1 by inhibiting oxidative stress and inflammatory responses and further reduce MyHC degradation, thereby alleviating denervated muscle atrophy.

Mitophagy refers to the process that selectively removes damaged or incomplete mitochondria *via* the mechanism of autophagy. In the human body, mitophagy maintains the integrity of mitochondrial function, thereby delaying aging and treating diseases (Wing et al., 2011). Gatica et al. (2018) showed that maintaining a balance in autophagy is particularly important in the body and that excessive autophagy can cause skeletal muscle atrophy. Another study indicated a significant increase in the expression of beclin 1 and LC3B in the skeletal muscle in spinal muscular atrophy (Sandri, 2010). Autophagy plays an important role in the process of spinal muscular atrophy and denervated muscle atrophy. Autophagy inhibition can effectively relieve muscle atrophy, and autophagy is a key target for the treatment of muscle atrophy (Piras et al., 2017; Cicardi et al., 2019; Wang et al., 2019; Cui et al., 2020). Our findings demonstrate that isoquercitrin can significantly inhibit mitophagy caused by denervation of the target muscle, accompanied by decreased expression of the autophagy-related proteins ATG7, BNIP3, and PINK1. Therefore, isoquercitrin treatment may prevent the occurrence of a process associated with autophagy inhibition as a part of its protective effect against denervated muscle atrophy. ROS may induce autophagy by activating the mucolipin-lysosome Ca^{2+}-transcription factor EB pathway (Zhang et al., 2016). Smuder et al. (2018) found that diaphragmatic mitochondrial ROS production during mechanical ventilation is essential to promote the expression of autophagy-related genes (such as *LC3*, *Atg7*, *Atg12*, *Beclin1*, and *p62*) and to increase the activity of cathepsin L, i.e., oxidative stress stimulates autophagy enhancement. In patients with chronic inflammation, inflammatory factors (IL-6, TNF-α, and TGF-β) can cause exacerbated mitophagy and dysfunction by reducing PGC-1α and upregulating autophagy-related genes (LC3B, Beclin-1, p62, Atg5, and Bnip3; Carson et al., 2016; VanderVeen et al., 2017). Our findings indicate that isoquercitrin can upregulate the expression of antioxidant-related genes in the target muscle following denervation, downregulate the expression of ROS production-related genes, inhibit inflammatory factors, and block the JAK/STAT3 pathway. Overall, isoquercitrin may inhibit autophagy in the target muscle following denervation by suppressing ROS and inflammatory signals, thereby inhibiting the expression of autophagy-related genes, reducing autophagy, and eventually protecting denervated muscle against atrophy.

Skeletal muscle comprises various fibers with different metabolic characteristics. Each muscle fulfills a specific function and responds differently to external stimuli and disturbances due to different innervations and fiber types (Lang et al., 2018). The conversion between muscle fiber types is a dominant feature of muscle atrophy. The slow-to-fast fiber type conversion severely impacts the function of the target muscle following denervation. In the present study, we found that isoquercitrin can delay the conversion from slow-to-fast muscle fibers following denervation, accompanied by a decrease in the expression of TnI-FS and an increase in the expression of TnI-SS. PGC-1α is a key factor that regulates mitochondrial function, modulates muscle fiber type, and controls fast-to-slow muscle fiber type conversion (Lin et al., 2002). Our findings revealed that isoquercitrin significantly inhibits the decreased expression of PGC-1α in denervated target muscles. Therefore, isoquercitrin may reverse the slow-to-fast fiber type conversion in denervated target muscles by promoting the expression of PGC-1α, thereby protecting against denervated muscle atrophy. Existing studies have shown that ROS production suppresses silent information regulator 1 (SIRT1)/PGC-1α signaling and that a decrease in ROS production can increase the expression of SIRT1 and PGC-1α proteins in the skeletal muscle of aging rats (Yu et al., 2018b; Yang et al., 2019). Moreover, inflammatory factors (IL-6, TNF-α, and TGF-β) can reduce the expression of PGC-1α (Carson et al., 2016; VanderVeen et al., 2017). The results obtained in our study indicate that isoquercitrin can inhibit oxidative stress and inflammation. Therefore, isoquercitrin can reduce autophagy and may inhibit oxidative stress and inflammation signals to promote the expression of PGC-1α following skeletal muscle denervation, by reversing the slow-to-fast fiber type conversion following denervation, thereby protecting against denervated muscle atrophy.

It should be mentioned that there are some limitations. In this study, we only studied the protective effect of isoquercetin on denervated soleus (slow-twitch fibers) muscle atrophy. Future studies to evaluate the effect of isoquercitrin on other muscles [e.g., TA (fast-twitch fibers) muscle] against denervation-induced muscle atrophy in mice are warranted. Pharmacokinetic study of isoquercitrin in plasma after intraperitoneal administration is a matter of significance, as well as the impact of isoquercitrin on contralateral leg. The young mice used in the experiments grew considerably during the period 14 days post nerve injury, which might induce some added impact compared to adult mice. Another drawback of our study was that all the analyses were done after 2 weeks of treatment, which do not allow to fine tune the molecular events underlying the process.

In summary, this study further confirms the role of oxidative stress and inflammatory response in denervated muscle atrophy. Isoquercitrin can alleviate denervated muscular atrophy by inhibiting oxidative stress and reducing inflammatory response to reduce autophagy, inhibit proteolysis *via* the ubiquitin-protease system, and suppress muscle fiber type conversion (**Figure 7**). Our findings enrich the knowledge regarding the molecular

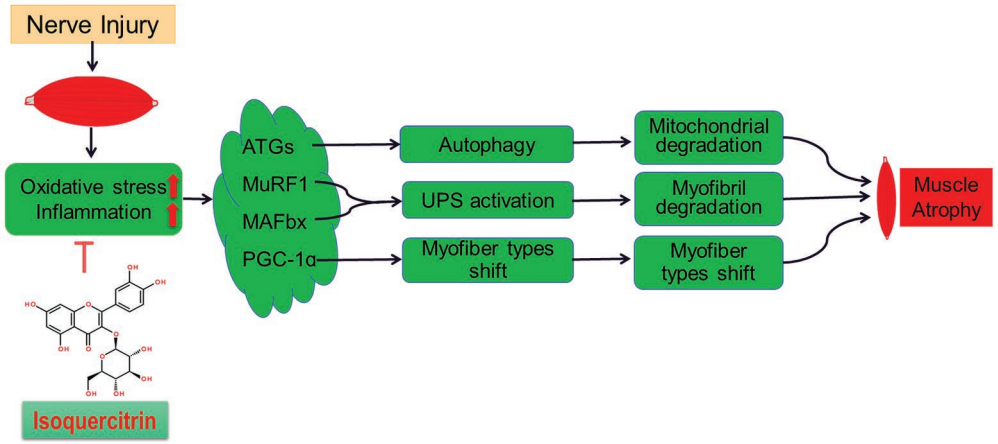

FIGURE 7 | A schematic diagram illustrating the proposed mechanism by which peripheral nerve injury induces soleus muscle atrophy. Denervation-induced skeletal muscle atrophy is associated with oxidative stress and inflammation. The inhibition of oxidative stress and inflammation through Isoquercitrin alleviated denervation-induced skeletal muscle atrophy by reducing proteolysis, inhibiting mitophagy, and reversing the slow-to-fast fiber type conversion following denervation.

regulation mechanism of denervated muscular atrophy and provide a scientific basis for the use of isoquercitrin as a protective drug for the prevention and treatment of muscle atrophy.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by the animal care guidelines of Nantong University and ethically approved by Jiangsu Administration Committee of Experimental Animals.

AUTHOR CONTRIBUTIONS

HS and FD designed the study. YS, ZH, QZ, WM, XY, and JZ performed the experiments. YS, ZH, QZ, WM, XY, and JQ collected and assembled data. ZH and WM performed data analysis. FD provided scientific expertise. HS wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.00988/full#supplementary-material.

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REVIEW
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The Impact of Immune Cells on the Skeletal Muscle Microenvironment During Cancer Cachexia

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Progressive weight loss combined with skeletal muscle atrophy, termed cachexia, is a common comorbidity associated with cancer that results in adverse consequences for the patient related to decreased chemotherapy responsiveness and increased mortality. Cachexia's complexity has provided a barrier for developing successful therapies to prevent or treat the condition, since a large number of systemic disruptions that can regulate muscle mass are often present. Furthermore, considerable effort has focused on investigating how tumor derived factors and inflammatory mediators directly signal skeletal muscle to disrupt protein turnover regulation. Currently, there is developing appreciation for understanding how cancer alters skeletal muscle's complex microenvironment and the tightly regulated interactions between multiple cell types. Skeletal muscle microenvironment interactions have established functions in muscle response to regeneration from injury, growth, aging, overload-induced hypertrophy, and exercise. This review explores the growing body of evidence for immune cell modulation of the skeletal muscle microenvironment during cancer-induced muscle wasting. Emphasis is placed on the regulatory network that integrates physiological responses between immune cells with other muscle cell types including satellite cells, fibroblast cells, and endothelial cells to regulate myofiber size and plasticity. The overall goal of this review is to provide an understanding of how different cell types that constitute the muscle microenvironment and their signaling mediators contribute to cancer and chemotherapy-induced muscle wasting.

Keywords: extracellular matrix, macrophage, satellite cell, fibroblast, endothelium

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INTRODUCTION

Cachexia is a perilous comorbidity occurring with many chronic diseases that are defined by progressive weight loss, skeletal muscle atrophy, and the inability to be fully prevented or treated by nutritional support (Fearon et al., 2011). Adverse consequences resulting from cachexia include loss of functional independence, poor treatment outcomes, and a reduced quality of life (Bruera, 1997, Fearon et al., 2011). Cancer cachexia is a complex condition that is the most common cancer-associated comorbidity and occurs in ~50–80% of cancer patients (Baracos et al., 2018). To this end, the cachectic condition is linked to reduced responsiveness to anticancer therapy and increased mortality (Kazemi-Bajestani et al., 2016; Baracos et al., 2018; Sealy et al., 2020). There is inherent difficulty to examining the mechanistic drivers of cachexia, namely a large

number of disruptions to systemic homeostatic regulation that occur with cancer, the inherent heterogeneity of different cancers, and the patient's underlying health condition. Moreover, skeletal muscle mass is sensitive to the several systemic disruptions associated with cancer including insulin resistance, chronic inflammation, anorexia, and hypogonadism (Baracos et al., 2018). There also has been significant interest in investigating tumor derived factors that can directly drive skeletal muscle catabolism, and numerous factors have been identified (Biswas and Acharyya, 2020). Despite our vastly improved mechanistic understanding of cancer-induced muscle wasting through established and well-defined preclinical cachexia and *in vitro* atrophy models, the complexity and heterogeneity of cancer cachexia have hindered the development of effective treatments for the cancer patient (Anderson et al., 2017). Additionally, mechanistic studies have not historically considered the potential additive effects of cancer and chemotherapy on the mechanisms inducing cachexia, and we are only beginning to understand the implications of this interaction for the management of cachexia (Barreto et al., 2016a,b; Bozzetti, 2020).

Systemic and local inflammation accompany many different conditions that produce skeletal muscle metabolic plasticity, growth, and atrophy, and a regulatory role for inflammation in these processes has been widely investigated for decades (Tidball, 1995; Deans and Wigmore, 2005). Additionally, transient increases in systemic inflammation and intrinsic skeletal muscle inflammatory signaling can occur with exercise and has been linked to many important muscle adaptations (Febbraio et al., 2004; Deyhle et al., 2015). Chronic systemic inflammation is a widely investigated driver of muscle wasting through its direct effects on skeletal muscle (Baracos et al., 2018), and its ability to induce other systemic disruptions that can ultimately regulate skeletal muscle mass, such as insulin resistance and hypogonadism (Wu and Ballantyne, 2017). The ability to regenerate from injury is a recognized property of healthy skeletal muscle, and immune cells have a well-established role in this regenerative process (Howard et al., 2020). While inflammation's contribution to initiating and accelerating cancer cachexia has been widely investigated (Evans et al., 2008; Carson and Baltgalvis, 2010), a major focus of this research has centered on circulating inflammatory mediators and how they directly regulate muscle intracellular signaling to disrupt protein turnover and metabolism to drive wasting (Talbert et al., 2018). To this end, significant gaps remain in our understanding of other aspects of the complex relationship between the immune system and the regulation of skeletal muscle mass. Additional research is warranted to delineate the capacity for inflammation to regulate signaling between different cell types in skeletal muscle that is involved in maintaining metabolic and protein turnover homeostasis. Immune cells comprise 2–6% of skeletal muscle's cell population, but maintain a well-established role in skeletal muscle homeostasis, especially macrophages (MΦ; Tidball, 2002; Reidy et al., 2019a). While the understanding of the MΦ's role in skeletal muscle repair and remodeling is well-appreciated, there is strong evidence for both T-cells and neutrophils in the maintenance of skeletal muscle MΦ function and overall skeletal muscle plasticity (Frenette et al., 2002; Tidball, 2005; Dumont et al., 2008; Schiaffino et al., 2017; Tidball, 2017; Deyhle and Hyldahl, 2018). Despite the importance of immune cell activity in muscle plasticity and aging (Reidy et al., 2019a), our understanding of immune cell involvement in cancer- and chemotherapy-induced muscle wasting is just emerging.

The potential for cancer to disrupt tightly regulated interactions between cell types in the skeletal muscle microenvironment continues to develop and be appreciated (Talbert and Guttridge, 2016). Skeletal muscle microenvironment interactions have established functions in muscle response to regeneration from injury, growth, aging, overload-induced hypertrophy, and exercise (Morgan and Partridge, 2020). Furthermore, there has been extensive investigation into the importance and regulation of satellite cell proliferation and differentiation, angiogenesis, and extracellular matrix (ECM) remodeling after muscle injury and with aging (Tidball and Wehling-Henricks, 2007; Xiao et al., 2016; Ceafalan et al., 2018; Yang and Hu, 2018). These adaptive processes are often coupled to local inflammatory responses initiated by remodeling stimuli. These inflammatory responses are subjected to precise temporal regulation and if this response is altered, muscle remodeling can be either attenuated or blocked (Howard et al., 2020). Moreover, systemic and intrinsic stimuli can induce MΦs to initiate signaling that regulates muscle fibroblasts, satellite cells, endothelial/vascular cells, as well as within the myofiber (Tidball, 2002; Arnold et al., 2007; Fry et al., 2014). Inflammatory signaling can impact several cell types located in the muscle microenvironment leading to altered myofiber protein synthesis (Gao et al., 2017) and mitochondrial quality control (Gomez-Cabrera et al., 2016; Fix et al., 2018), which are known drivers of muscle wasting with cancer. Additionally, muscle fibrosis and dysregulation of the ECM, of which immune cells play a central regulatory role, have been reported in cachectic skeletal muscle from pancreatic cancer patients (Judge et al., 2018; Nosacka et al., 2020). Therefore, this review explores the growing body of evidence for immune cell modulation of the skeletal muscle microenvironment during cancer-induced muscle wasting. Emphasis is placed on the regulatory network that integrates physiological responses between immune cells with other muscle cell types including satellite cells, fibroblast cells, and endothelial cells to regulate myofiber size and plasticity. The overall goal of this review is to provide an understanding of the potential for different cell types that constitute the muscle microenvironment that can contribute to cancer-induced muscle wasting. Specific attention is given to MΦ, neutrophil, and T-cell's regulation of the communication between several cell types in the muscle microenvironment that could promote cancer-induced myofiber catabolism and metabolic dysfunction. The current understanding of chemotherapy as an underlying pathology that could disrupt immune cell interaction with the skeletal muscle microenvironment also is discussed.

CANCER AND CHEMOTHERAPY-INDUCED CACHEXIA OVERVIEW

Mechanisms of Cancer-Induced Cachexia

Skeletal muscle loss is a critical manifestation of cancer cachexia (Argiles et al., 2010). While our mechanistic understanding of cancer-induced muscle wasting continues

to develop and potential therapeutic targets to treat the condition have been identified, both the complexity of the underlying disease and the multiple mechanisms responsible for the maintenance of skeletal muscle homeostasis have clouded our ability to understand the primary drivers of this catabolic condition. While there may still be hope for a "master regulator" of cancer-induced muscle atrophy (Lecker et al., 2004), the heterogeneity of cancer, pre-existing conditions of the patient, and equivocal findings between preclinical cachexia models have led to a host of potential drivers of the cachectic condition that are too broad to describe in detail here. However, this topic has been extensively reviewed (Argiles et al., 2014, 2015; Carson et al., 2016; Vanderveen et al., 2017; Baracos, 2018). Skeletal muscle responds to a multitude of systemic cues that are vital for whole body homeostasis, and the muscle fiber responds to global stimuli including disuse, increased activity, aging, and metabolic mediators (Atherton et al., 2016), which can all be altered with cancer and chemotherapy. Furthermore, due to these varied physiological and functional demands, muscle fibers differ in contractile and metabolic properties, and these properties can impact how the muscle fiber responds to the cancer environment (Carson et al., 2016; Vanderveen et al., 2017). The complexity of the muscle's physiological response to cancer and chemotherapy is also impacted by the myofiber's interaction with the ECM, vasculature, and response to local inflammation, which is examined in this review.

Despite the identified complexities in understanding muscle mass regulation with cancer, muscle mass maintenance requires a balanced regulation of protein turnover, which is maintained by anabolic and catabolic signaling pathways. Cancer induces fundamental disruptions to the homeostatic regulation of the protein turnover that has negative consequences for skeletal muscle function and cellular metabolism (Baracos et al., 2018; Counts et al., 2020). Cachexia drives overall muscle mass loss even with adequate nutrient intake (Fearon et al., 2012a; Porporato, 2016), and results in suppressed protein synthesis and/or increased degradation. Muscle protein synthesis and breakdown can be regulated by various stimuli including nutrient availability, hormones, mechanical loading, and metabolic stress, which can all be disrupted by cancer and potentially chemotherapy (Glass, 2005; Powers et al., 2005; Zhang et al., 2007; Egerman and Glass, 2014). Active areas of investigation for the disruption of protein turnover with cancer have focused on excessive protein breakdown by the ubiquitin proteasome system (UPS) and disrupted autophagy regulation (McClung et al., 2010; White et al., 2011; Fearon et al., 2012b; Penna et al., 2013; Luo et al., 2014; Aversa et al., 2016). Critical intracellular regulator networks in skeletal muscle exert tight control of these processes but can be disrupted by cancer and chemotherapy. This skeletal muscle regulatory nexus involves Akt, mTORC1, FOXO3A, and AMPK signaling, which responds to growth factors, mechanical stimuli (i.e., stretch and contraction), cellular energy levels (AMP: ATP), nutrients (amino acids), and inflammation (Carson, 1997; Carson and Wei, 2000; Glass, 2005; Judge et al., 2014). These mechanisms exert control over cell growth through regulation of protein synthesis (e.g., transcription and translation), protein degradation (e.g., UPS) autophagy, and metabolism (Laplante and Sabatini, 2009). Disruption to this signaling network in cachectic skeletal muscle also has been linked to cancer-induced mitochondrial dysfunction (Carson et al., 2016; Vanderveen et al., 2017, 2019; Fix et al., 2019). Muscle mitochondrial dysfunction continues to be extensively investigated as an underlying mechanism for the initiation and progression of cancer-induced muscle wasting. However, understanding how cancer disrupts the normal dynamic regulation of muscle protein turnover remains an active area of inquiry.

Cancer-induced systemic inflammation and the increased production of specific cytokines have been extensively investigated as an underlying driver of cancer cachexia (Argilés et al., 2019). Both adipose tissue and skeletal muscle catabolism can be induced by exacerbated inflammation. Examination of cachectic cancer patients and preclinical models of cachexia has identified interleukin 6 (IL-6), tumor necrosis factor α (TNF-α), TNF-like inducer of apoptosis (TWEAK), TNF receptor (TNFR)-associated factor 6 (TRAF6), interferon gamma (INF-γ), and leukemia inhibitory factor (LIF) as mediators of cancer-induced muscle wasting (White et al., 2013; Hetzler et al., 2015; Zimmers et al., 2016; Yakovenko et al., 2018; Argilés et al., 2019). These cytokines can induce several intracellular pathways including the nuclear factor-κB (NF-κB) pathway, p38 mitogen-activated protein kinase (MAPK) pathway, and the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway. Notwithstanding the identification of several different inflammatory mediators in cachexia, we lack understanding of how these signaling pathways interact or overlap with other physiological signaling within the muscle microenvironment to regulate muscle catabolism with cancer. Due to this complexity barriers remain for implementing therapeutic strategies that target an individual signaling cascade to preserve skeletal muscle mass in the cancer patient. Moreover, there are currently no FDA-approved treatments for cancer cachexia. That said, there remains strong therapeutic potential for nutritional, anti-inflammatory, and lifestyle management strategies to mitigate cachexia severity and improve patient life quality.

Mechanisms of Chemotherapy-Induced Cachexia

Cachexia's prevalence with cancer is often dependent on cancer type and stage of the disease; however, the functional deficits with chemotherapy are consistent and pervasive (Barreto et al., 2016a; Bozzetti, 2020). Along with nausea, emesis, and anorexia, weakness and fatigue remain among the most commonly reported off-target effects of chemotherapy (Dahele et al., 2007). Our understanding of chemotherapy's deleterious off-target effects on skeletal muscle has improved over the last decade highlighting a key role for metabolic and DNA/cell stress (Gilliam et al., 2009; Barreto et al., 2016b; Morton et al., 2019; Sougiannis et al., 2019). Targeting the generation of mitochondrial reactive oxygen species (ROS), by exercise or antioxidants, has shown promise in mitigating Doxorubicin (DOX)-induced skeletal muscle dysfunction (Smuder, 2019; Montalvo et al., 2020), and targeting the activation of MAPKs with ACVR2B/Fc and MEK1 inhibitors has been proposed to alleviate 5-FU

induced mitochondrial dysfunction (Barreto et al., 2016b, 2017). Inflammatory signaling has been demonstrated to regulate chemotherapy-induced E3 ligase activation through modulating TNF-α (Gilliam et al., 2009). Interestingly, while conclusive mechanisms involving intrinsic skeletal muscle inflammatory signaling with chemotherapy are lacking, leukopenia with chemotherapy has been well-established (Kvinnsland, 1999; Yamanaka et al., 2007; Shitara et al., 2009; Baechler et al., 2010; Han et al., 2012; Abraham et al., 2015). This naturally invites intrigue into understanding how chemotherapy-induced immunosuppression can converge with cancer-associated chronic inflammation to disrupt the skeletal muscle microenvironment, which can lead to wasting, reduced life quality, poor treatment outcomes, and subsequent reduced survival. While again there are currently no approved treatments for chemotherapy's off-target effects on muscle, there remains similar strong therapeutic potential for nutritional, anti-inflammatory, antioxidant, and/or lifestyle management to mitigate chemotherapy-induced toxicities.

The identification of immune checkpoints, specifically PD-1/PDL1 and CTLA-4, has led to the use of specific inhibitors, which have greatly improved cancer therapies (Haanen and Robert, 2015). These drugs improve the anti-tumor capacity of T-cells and potentially natural killer (NK) cells by blocking the ligand/receptor binding induced by cancer cells that cause T-cell inactivation (Curran et al., 2010; Pesce et al., 2019). Similar to other cancer treatments, patients receiving immune check point inhibitors (ICPIs) that suffer from cachexia have reduced survival when compared to patients who maintain their weight (Chu et al., 2020; Roch et al., 2020). Whether ICPIs can induce cachexia has not been established; however, body weight loss in aged mice with mesothelioma tumors was exacerbated by immunotherapy, but whole body macrophage depletion reduced tumor mass and body weight loss (Duong et al., 2018). As using ICPIs become increasingly more common as a cancer treatment, understanding the impact of cachexia and the role of ICPI in cachexia progression remains an intriguing area for future inquiry.

Overlap and Differences of Cancer and Chemotherapy-Induced Cachexia

Developing an understanding of the distinct contributions of cancer and chemotherapy to muscle wasting and dysfunction has been at the forefront of cachexia related mechanistic inquiry; however, understanding the potential synergism or negation of specific mechanisms with both cancer and chemotherapy would improve our understanding of cancer patient's condition. To date, few studies have directly investigated the effect of cancer and chemotherapy on skeletal muscle which leads to cachexia. Barreto et al. (2016a) identified the common pathways activated by C26-induced cachexia and chemotherapeutic Folfiri [5-fluorouracil (5FU), leucovorin, irinotecan]-induced cachexia. This study highlighted a common downregulation of metabolic and structure proteins with particularly striking changes to mitochondrial function (Barreto et al., 2016a). Interestingly, only Folfiri induced neurological damage, while only C26 was associated with exercise intolerance. Others have demonstrated that TNF-α plays a key role in cancer and DOX-induced skeletal muscle dysfunction (Hardin et al., 2008; Gilliam et al., 2009, 2011). While disrupted protein turnover has been a central regulator of cancer-induced cachexia (White et al., 2013; Montalvo et al., 2018; Counts et al., 2020), there is little evidence to suggest chemotherapy impacts muscle in this same way.

IMMUNE CELLS

Monocytes

Monocyte's Role in Physiology

The mononuclear phagocyte system (MPS) includes circulating monocytes, monocyte derived MΦs, and dendritic cells (DC; Gordon and Taylor, 2005; Hume, 2006). As their name implies, they share a mononuclear structure and play key roles in tissue defense and homeostasis through phagocytosis of old or damaged materials as well as invading pathogens (Murphy and Weaver, 2016). Upon insult, naïve monocytes proliferate in the bone marrow in response to chemokines, most notably monocyte chemoattractant 1 (MCP-1), to infiltrate the damaged/infected tissue to phagocytize pathogens or damaged material (Hume, 2006; Capoccia et al., 2008). Once outside vasculature, monocytes most commonly differentiate to either DC or MΦ; however, tissues also house resident, nascent, and self-renewing DCs and MΦs (Van Furth and Cohn, 1968; Hoeffel and Ginhoux, 2018). It is also important to note that MΦs have been demonstrated to play both an anti- and pro-inflammatory roles in both innate and adaptive immunity depending on the stimuli; however, MΦs are the primary phagocytic effector cell of the MPS (Gordon and Taylor, 2005). While DCs share a morphology with undifferentiated monocytes and MΦs, they primarily function as antigen presenting cells (APC) which activate naïve T-cells within the adaptive immune response (Mellman, 2013; Murphy and Weaver, 2016; Abbas et al., 2018; **Figure 1**).

Unfortunately, due to overlapping functions and shared cell surface markers, characterizing and assessing the different subsets of these monocytes are contentious and equivocal (Gordon and Taylor, 2005; Guilliams et al., 2014). The most conserved monocyte cell surface marker between humans and rodents are CX3C chemokine receptor (CX_3CR1) and cluster of differentiation (CD) 11b. These markers vary in expression based on the phenotype of the monocyte. For example, resident or "patrolling" monocytes will have a higher expression of CX_3CR1 compared to circulating or inflammatory monocytes, and the expression of Ly6C in mice is a spectrum from resident (low-none) to inflammatory or circulating (med-high; Gordon and Taylor, 2005). However, undifferentiated $Ly6C^{High}$ monocytes have been described in healthy uninjured tissue while $Ly6C^{Low}$ survey the endothelium (Guilliams et al., 2014). DCs share the CX_3CR1 and CD11b receptors with other monocytes; however they differ in their expression of CD11c and major histocompatibility complex (MHC) II and can even express common T-lymphocyte markers CD4 and CD8 (Sato and Fujita, 2007). The varying degrees of these cell surface markers help define physiology and function as well as their myeloid or lymphoid origin/maturation (Sato and Fujita, 2007; Mellman, 2013). All murine MΦs are said to express CD68 and F4/80 irrespective of phenotype.

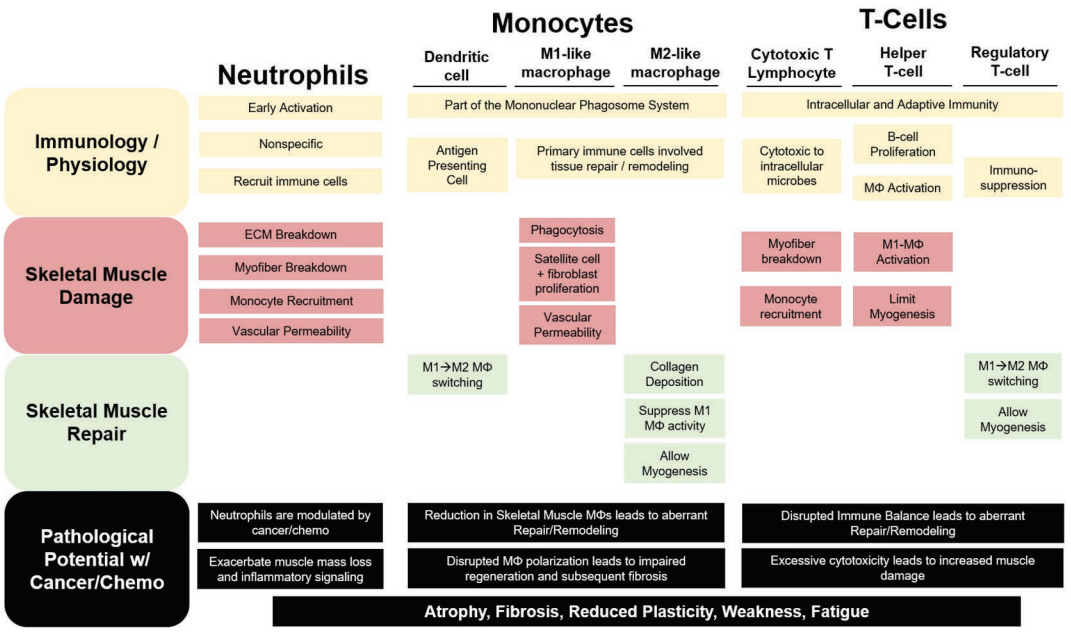

FIGURE 1 | The contribution of immune cells to skeletal muscle repair and remodeling. Neutrophils, monocytes, and T-cells have both unique and redundant processes that aid in resolving a skeletal muscle insult. Neutrophils are recruited early and exact a robust, non-specific response that can exacerbate damage to the myofiber and extracellular matrix (ECM). Cytotoxic T-lymphocytes (CTLs) have been demonstrated to perform similar functions to neutrophils in the early stage of the damage response. Additionally, Neutrophils and CTLs recruit inflammatory (M1-like) macrophages (MΦ) to help phagocytize damaged tissue for removal while releasing pro-inflammatory mediators to stimulate satellite and fibroblast cell proliferation and increase vascular permeability. As repair progresses anti-inflammatory, profibrotic (M2-like), MΦs predominate skeletal muscle allowing for myogenesis and collagen deposition. Regulatory T-cells and dendritic cells (DC) aid in the MΦ phenotype switching contributing to the switch from a pro-inflammatory to an anti-inflammatory skeletal muscle microenvironment. Cancer and chemotherapy have been demonstrated to modulate each of these immune cells uniquely in circulation or other non-muscle tissue; however, we are limited in our understanding of their roles in cancer-associated wasting. We can speculate that these disruptions to these cells within the skeletal muscle microenvironment would lead to muscle mass loss and increased fibrosis contributing to reduced muscle plasticity, weakness, and fatigue.

Mature/differentiated MΦs are not common in the circulation but are found as naïve monocytes until they extravasate. Resident self-renewing MΦs are commonly considered M0 and lack, or have low expression of CD11c, MHCII, CD80, or CD206. These resident M0-like MΦs can either remain nascent or be polarized to increase expression of CD11c and/or MHCII to become a M1-like pro-inflammatory, phagocytic MΦ (Shapouri-Moghaddam et al., 2018; Orecchioni et al., 2019). While these resident cells are able to polarize to an M1-like phenotype, this classification is more common among infiltrating monocytes ($Ly6C^{High}$). Furthermore, these M1-like MΦs are plastic and their expression of pro-inflammatory cell surface markers and release of pro-inflammatory cytokines can be suppressed with a corresponding increase in the expression of CD206 and/or CD163 and release of anti-inflammatory cytokines resulting in an M2-like anti-inflammatory, pro-fibrotic MΦ (Gordon and Taylor, 2005; Mosser and Edwards, 2008; Wynn et al., 2013). MΦs are plastic and responsive to extrinsic stimuli; however, autocrine/paracrine MΦ signaling can regulate their phenotype through feedback or feedforward mechanisms. Furthermore, the M1/M2 dichotomy does not adequately described the nature and function of the MΦ and should be considered a spectrum rather than an "either/or" (Mosser and Edwards, 2008). This classification of cell surface markers and function is not exhaustive and is often contentious, which has been previously reviewed (Hume, 2006; Mellman, 2013; Guilliams et al., 2014).

Monocyte's Role in Skeletal Muscle Repair and Remodeling

MΦs have received significant attention in skeletal muscle physiology given their central role in repair and remodeling, and thus our understanding of skeletal muscle MΦs primarily comes from studies investigating repair, regeneration, and remodeling following unloading/reloading-, eccentric contraction-, or cardiotoxin-induced damage (St Pierre and Tidball, 1994; Tidball, 2005, 2017; Cote et al., 2013). Early in skeletal muscle repair, there is an influx of circulating monocytes that differentiate to a phagocytic and pro-inflammatory M1-like MΦ to aide in the removal of damaged tissue. Additionally, they recruit additional inflammatory monocytes by MCP-1, promote vascularization by HIF-1α, stimulate satellite cells proliferation, and inhibit satellite cells differentiation by IL-6 and TNF-α (Frenette et al., 2003; Arnold et al., 2007; Capoccia et al., 2008; Novak et al., 2014; Liu et al., 2017; Ceafalan et al., 2018). The plasticity of MΦs, however, allows for phenotypic flexibility and within 7 days following damage a more M2-like profibrotic anti-inflammatory phenotype predominates (St Pierre and Tidball, 1994; Tidball and Wehling-Henricks, 2007; Hammers et al., 2015). These M2-like MΦs then promote angiogenesis by secreting

vascular endothelial growth factor (VEGF) and ECM remodeling by secreting TGFβ and matrix metalloproteases (MMP) 2 and 9 while allowing for myogenesis through suppression of TNF-α release (De Santa et al., 2018).

Disrupted skeletal muscle repair with aging and chronic disease has been described, though mechanistic explanations often focus on sex hormones, satellite cells, or intrinsic structural dysmorphias. Despite the understood immune changes with aging and disease and the established roles of monocytes in skeletal muscle homeostasis, our understanding of the relationship between the immune system and musculoskeletal system under such conditions is in its infancy (Cui et al., 2019; Reidy et al., 2019a). Immunological investigation into muscular dystrophy demonstrated that dystrophic mice had profound increases in total muscle MΦs and targeting this infiltration can mitigate disease progression (Mojumdar et al., 2014; Villalta et al., 2015). Additionally, reloading aged skeletal muscle had a blunted hypertrophy response associated with a lower number of M1-like MΦs at baseline and blunted M1-like MΦ infiltration (early) and M2-like MΦ transition (late; Reidy et al., 2019b). To this end, it is important to note that the mean age of cancer patients is ~65 years and it may be that these changes with aging overlap with cancer and chemotherapy-induced changes to skeletal muscle MΦs (Dunne et al., 2019).

Monocytes Role in Cancer and Chemotherapy-Induced Cachexia

To date, few papers have directly investigated the changes to skeletal muscle monocytes or MΦs with cancer and/or cancer-associated wasting (Inaba et al., 2018; Costamagna et al., 2020). Interleukin-4 (IL-4), a potent stimulator of M2-like MΦs, was shown to attenuate skeletal muscle wasting in the C26 mouse model of cachexia associated with reduced satellite cell accumulation and increased skeletal muscle CD206 protein expression (Costamagna et al., 2020). Protein analysis showed no significant differences between control and C26-tumor bearing mice with F4/80, and CD206 (marker of M2-like MΦs) appears lower in C26 mice but did not achieve statistical significance. While elevated circulating IL-6 and LIF is diagnostic in the C26 model, Inaba et al. (2018) demonstrated reduced skeletal muscle MΦ and neutrophil number without the loss of satellite cell proliferative or differentiative abilities that others have demonstrated (Penna et al., 2010). Evidence of disrupted skeletal muscle regeneration – a process reliant on skeletal muscle MΦs – with cachexia has been demonstrated (Devine et al., 2015; Judge et al., 2018). Whether cachexia leads to muscle degeneration or fibrosis without a damaging insult is equivocal likely due to the heterogeneity of tumor type and preclinical model; however, reduced specific force (force per unit area) with cachexia has been demonstrated across several preclinical models, corresponding with the severity of weight loss (Jaweed et al., 1983; Gorselink et al., 2006; Murphy et al., 2012; Roberts et al., 2013; Ramage and Skipworth, 2018; Vanderveen et al., 2018). The role of monocytes/MΦs in these aberrations has been merely speculative highlighting a need for additional studies examining changes to MΦs in skeletal muscle with cachexia progression. There is, however, intriguing evidence suggesting the immunosuppressive nature of common chemotherapeutic DOX may be disrupting skeletal muscle MΦ activation and polarization leading to blunted skeletal muscle repair and remodeling (Huang et al., 2017). Interestingly, disrupted skeletal muscle inflammatory signaling with DOX and/or 5FU has not been evident, despite the cytotoxic nature of the drugs. Current evidence remains focused on DNA damage or stress sensors, p38 and Erk1/2, mitochondrial damage, oxidative stress, and autophagy with these chemotherapeutics (Barreto et al., 2016a; Ballaro et al., 2019; Hiensch et al., 2019; Bozzetti, 2020).

Neutrophils

Neutrophils Role in Physiology

Neutrophils belong to a class of leukocytes characterized by their polymorphic nuclear shape and their early recruitment during acute inflammation (Kolaczkowska and Kubes, 2013; Stackowicz et al., 2019). Neutrophils make up roughly ~50% of circulating leukocytes in healthy individuals (~15% in mice) and are the first line of defense against bacterial infection (Kolaczkowska and Kubes, 2013). Interestingly, neutrophils share a precursor cell with monocytes and originate in the bone marrow but rely on granulocyte colony-stimulating factor (G-CSF) for simulation as opposed to monocyte colony-stimulating factor (M-CSF). Classifying neutrophils is much more straight forward compared to monocytes; however, there remains variety among immunologists. Neutrophils commonly express CD11b and CD16 (Fujimoto et al., 2000); however, recently the use of Ly6G and Gr-1 has been used to differentiate neutrophils from other leukocytes (Lee et al., 2013; Romee et al., 2013). Neutrophils kill/eliminate pathogens through phagocytosis, degranulation, ROS production, and/or neutrophil extracellular traps, which have been well-reviewed (Kolaczkowska and Kubes, 2013; Mayadas et al., 2014). The granules within neutrophils contain lysozymes, collagenases, and elastases, which serve the function of damaging/killing foreign material. Unfortunately, neutrophils are early and robust in their response and their lack of specificity often damages the host tissue along with the pathogen (Faurschou and Borregaard, 2003; Mayadas et al., 2014). While they play an important role in acute cytotoxicity, neutrophils release a myriad of cytokines and oxidative factors to initiate and potentiate the immune response depending on the stimuli. Neutrophils are often replaced by MΦs following the initial stages of damage or infection primarily through neutrophil release of pro-inflammatory cytokines and myeloperoxidases (MPOs).

Neutrophils Role in Skeletal Muscle Repair and Remodeling

The majority of studies investigating the musculoskeletal and immune system relationship have focused on monocytes/MΦs; however, there is evidence that neutrophils play a key role in the clearance of damaged/dead tissue and recruitment of MΦs (Tidball et al., 1999; Pizza et al., 2005). Historically, ischemia/reperfusion-induced skeletal muscle damage resulted in a dramatic increase influx of neutrophils while MΦs play a more minor role (Korthuis et al., 1988; Smith et al., 1989; Kanwar et al., 1998); however, the role for neutrophils in eccentric

contraction-induced damaged also has been described (Frenette et al., 2002; Pizza et al., 2005). While the plasticity of MΦ polarization is a key regulator of the switch from the initial skeletal muscle damage response to repair/regeneration, neutrophils appear to play a significant role only in the initial damage response. To this end, persistent neutrophil infiltration has been demonstrated to delay skeletal muscle repair and absence of neutrophil accumulation mitigates signs of skeletal muscle damage (Pizza et al., 2005). Further, elevated neutrophils have been demonstrated to exacerbate joint damage with arthritis, and some evidence suggests that neutrophils can induce or exacerbate skeletal muscle damage (Dumont et al., 2008). The induction of neutrophils with exercise and ischemia/reperfusion-induced damage is transient, and the decrease in the relative abundance of neutrophils is accompanied by a polarization switch of MΦs to an M2-like profibrotic/anti-inflammatory phenotype for remodeling (Tidball et al., 1999; Frenette et al., 2003; Tidball, 2005; Tidball and Villalta, 2010). Interestingly, the acute changes in the genetic profile of skeletal muscle with exercise reflect similar changes in blood neutrophils and circulating inflammatory cytokines (Broadbent et al., 2017). While the role of neutrophils in skeletal muscle physiology requires significant work, there is evidence to support that neutrophils exacerbate the damage response to perpetuate the acute inflammatory stimuli necessary for repair and remodeling.

Neutrophils Role in Cancer and Chemotherapy-Induced Cachexia

Neutropenia has been shown to be a strong prognostic indicator for survival in cancer patients undergoing chemotherapy (Kvinnsland, 1999; Yamanaka et al., 2007; Han et al., 2012; Abraham et al., 2015). Regarding cancer, the circulating neutrophil-to-lymphocyte ratio (NLR) is also a strong prognosticator for all-around survival (Grecian et al., 2018). In general, tumors produce G-CSF and can increase circulating neutrophils (Jablonska et al., 2017) while chemotherapy reduces circulating neutrophils (Shitara et al., 2011). These findings appear dependent on cancer type and chemotherapy as some investigators have reported decreases in neutrophils with cancer and increases with chemotherapy. To our knowledge, the impact of neutrophil changes with cancer and chemotherapy on skeletal muscle has not been investigated. We can speculate, however, that an increase in circulating neutrophils with cancer may initiate and/or potentiate skeletal muscle degeneration or mass loss while neutropenia may blunt the homeostatic balance of skeletal muscle repair and remodeling. It is understood that a proper balance of pro- and anti-inflammatory effectors is essential for skeletal muscle homeostasis and the undulating changes to neutrophils with cancer and cancer therapies warrants investigative inquiry.

Lymphocytes: T-Cells

T-Cells Role in Physiology

Lymphocytes are key regulators of the immune response and play a central role in adaptive immunity. Lymphocytes are characterized as B (B-cells) or T lymphocytes (T-cells) based on their function and origin (B-Bone Marrow; T-Thymus) rather than their appearance as they both maintain a similar morphology. Similar to other immune cells, B-cells release inflammatory mediators to regulate an immune response, but their primary function is often focused on extracellular pathogens and antibody production (Murphy and Weaver, 2016). Their role as an immune modulator with skeletal muscle has not been well-characterized and requires additional work; therefore, this review will focus on the known roles of T-cells. T-cells are key regulators of cellular immunity either through eliminating intracellular microbes or the activation of other immune cells including MΦs (Abbas et al., 2018). The classification of T-cells is extensive and depends again on their cellular function and role in the immune response. Classic characterization aligns with CD3+CD4+ helper T-cells (Th), CD3+CD4+CD25+FoxP3+ T regulatory cells (Tregs), and CD3+CD4-CD8+ cytotoxic T lymphocytes (CTL). Historically, Th cells regulate B-cell differentiation in immune tissue and activate resident or local MΦs for cellular immunity and can be further classified into Th1, Th2, and Th17 (Th3, Th9, Th22, and TFH cells have also been described; Hirahara and Nakayama, 2016). Th1 cells regulate intracellular phagocytosis by activating MΦs through the release of pro-inflammatory IFNγ, while Th2 cells can release IL-4 and IL-13 to modulate the MΦ phenotype switching to an M2-like anti-inflammatory phenotype (Shapouri-Moghaddam et al., 2018; Mazzoni et al., 2019). Th17 cells are only beginning to be understood; however, they produce known pro-inflammatory cytokines IL-17 and IL-6, and have an emerging role in auto-immunity (Bi et al., 2007; Mazzoni et al., 2019). CD8+ CTL are aptly named as they provide intracellular immunity against pathogens by inducing cell death in cells that are not accessible or recognized by antibodies (Mittrucker et al., 2014). While Th cell activation and proliferation play a key role in antibody-mediated immune function in circulation, CTLs are the primary effectors of the intracellular immune response (Abbas et al., 2018). Tregs, as their name suggests, play a key role in regulating the immune response primarily through immune suppression and regulate the M1-like to M2-like MΦ phenotype switch during remodeling (Hori et al., 2003). The different subsets of lymphocytes are vast, and each play an important role in the immune system; however, for our purposes we will highlight the literature examining the roles of Th, Tregs, and CTLs in skeletal muscle physiology, repair, and remodeling and their potential role in modulating muscle wasting with cancer and chemotherapy.

T-Cells Role in Skeletal Muscle Repair and Remodeling

The role of T-cells in skeletal muscle repair and remodeling has been recently reviewed and described briefly below (Schiaffino et al., 2017; Deyhle and Hyldahl, 2018). Tregs primarily function in immune suppression, which is necessary during the later stages of skeletal muscle repair/remodeling, while CTLs and Th cells may potentiate the early damage response (Zhang et al., 2014; Deyhle and Hyldahl, 2018). Tregs release anti-inflammatory cytokine IL-10 to regulate M1-to-M2 like MΦ phenotype switching during muscle repair following injury (Deng et al., 2012; Schiaffino et al., 2017; D'Alessio et al., 2019).

Tregs also have been shown to stimulate proliferation of satellite cells through the release of Amphireglin (AREG) – a key regulator of immunity and tissue repair (Burzyn et al., 2013; Arpaia et al., 2015; Castiglioni et al., 2015; Zaiss et al., 2015). The mechanisms of Treg's regulation of skeletal muscle repair are still being unearthed; however, the induction of IL-33 with injured skeletal muscle appears the likely candidate for the increased abundance of Tregs and Treg-associated AREG (Kuswanto et al., 2016). Our understanding of CD8+ CTLs role in skeletal muscle repair centers around potentiating the immune response through its own infiltration into damaged muscle and releasing pro-inflammatory cytokines, namely MCP-1 (Zhang et al., 2014). Rag1 deficient mice that lack both T- and B-cells, but maintain intact MΦs, had delayed skeletal muscle repair following cardiotoxin; however, reintroduction of both CD4+ and CD8+ T-cells rescued the repair process timeline (Fu et al., 2015). These investigations primarily focused on the impact of T-cells on the activation and proliferation of satellite cells, which is necessary for the repair process.

T-Cells Role in Cancer and Chemotherapy-Induced Cachexia

CTLs and Th cells appear to play a role in the pathological damage response in Duchenne's muscular dystrophy (DMD), while there is promise for manipulating Tregs to mitigate excessive skeletal muscle damage (Spencer et al., 2001; Villalta et al., 2015). Similar to neutrophils, there has not been investigative inquiry into the role of T-cells in cancer or chemotherapy-induced skeletal muscle wasting; however, T-cells have been investigated extensively for their role in immune suppression during cancer progression and are promising therapeutic targets given their role in cytotoxicity/cellular immunity (Naito et al., 1998; Almand et al., 2001). To this end, tumor infiltrating lymphocytes (TILs) and *ex vivo* expansion of T-cells provides important prognosis information (Walsh et al., 2005; Morgan et al., 2006). Interestingly, CD8+ T-cells have been demonstrated to induce skeletal muscle wasting during infection-associated cachexia as shown by improved body weight maintenance in CD8+ T-cell null mice (Baazim et al., 2019). However, mechanisms underlying infection-associated cachexia may be distinct from cancer-associated cachexia. The most promising study was conducted by Wang et al. (2008) showing infusion of $CD4+CD44^{Low}$ naïve T-cells could attenuate muscle mass loss associated with ameliorating cancer-associated lymphopenia. Additionally, there is evidence of an inverse relationship between body weight and NLR – as NLR increases, body weight decreases – highlighting that this ratio may be an intriguing surrogate for the immune milieu responsible for cachexia progression (Derman et al., 2017). Given the important role of T-cells in cancer progression, treatment, and proposed role in muscle mass maintenance, studies aimed at understanding each T-cell's role in cancer and chemotherapy-associated cachexia are needed.

Other Immune Cell Modulators

While the primary focus of this review is to highlight the impact of monocytes, neutrophils, and T-cells on the skeletal muscle microenvironment, other immune cells involved in cancer's immune disruptions should not be neglected as their role in skeletal muscle homeostasis is merely less understood. These cells include, but are not limited to, B-cells, NK cells, myeloid derived suppressor cells (MDSC), and mast cells (Murphy and Weaver, 2016). The role of these various cell types in the pathogenesis of DMD has been reviewed (Madaro and Bouche, 2014). B-cells were recently shown to modulate muscle weakness during dermatomyositis through the regulation of IFNγ (Radke et al., 2018). Mast cells aggregate and proliferated in sites of muscle necrosis following damage and can restore vascular permeability during myositis (Gorospe et al., 1996; Yokota et al., 2014); however, little is known about their role with muscle wasting with cancer and chemotherapy. Interestingly, MDSCs were shown to be increased with cancer thought to contribute to global disruptions in energy metabolism contributing to cachexia progression (Cuenca et al., 2014). A correlative increase of IL-17 and MDSCs were both associated with nutritional impairments with gastrointestinal cancer which was proposed to contribute to cachexia (Yazawa et al., 2013). Furthermore, the metabolic cost of myelopoiesis, mainly increased MDSCs, may also contribute to the metabolic abnormalities seen with cancer and chemotherapy (Sica and Strauss, 2017).

SKELETAL MUSCLE MICROENVIRONMENT

Myofiber Overview

The myofiber response to cancer and the cachectic environment is considered to be central to understanding muscle wasting regulation. However, there is an emerging consideration for tumor derived factors and inflammatory mediators to go beyond targeting the myofiber directly (Biswas and Acharyya, 2020). The cachectic environment has the potential to also indirectly disrupt myofiber homeostasis through the altered regulation of other cell types in the muscle microenvironment (Talbert and Guttridge, 2016), highlighting a need to delineate the capacity for cancer and chemotherapy to disrupt myofiber interactions with other cell types in muscle (**Figure 2**). Furthermore, each cell type has a critical function within the skeletal muscle microenvironment, involving ECM remodeling and angiogenesis, which are established components of muscle plasticity in response to regeneration from injury, growth, aging, overload-induced hypertrophy, and exercise (Dennis et al., 2009; Morgan and Partridge, 2020). Since skeletal muscle fiber nuclei are post-mitotic, the role of the satellite cell in aging and overload hypertrophy has been actively investigated for over 30 years. This line of inquiry includes the role of myonuclear apoptosis in sarcopenia and myonuclear accretion in hypertrophy myofibers (McCarthy et al., 2011; Fry et al., 2014). As skeletal muscle remodeling is a critical property of muscle and is thought to be a continuous process over an individual's life span, there is growing interest in how aging, disuse, cancer, and chemotherapy treatments can disrupt remodeling processes in skeletal muscle, which could serve to exacerbate the development and progression of cachexia.

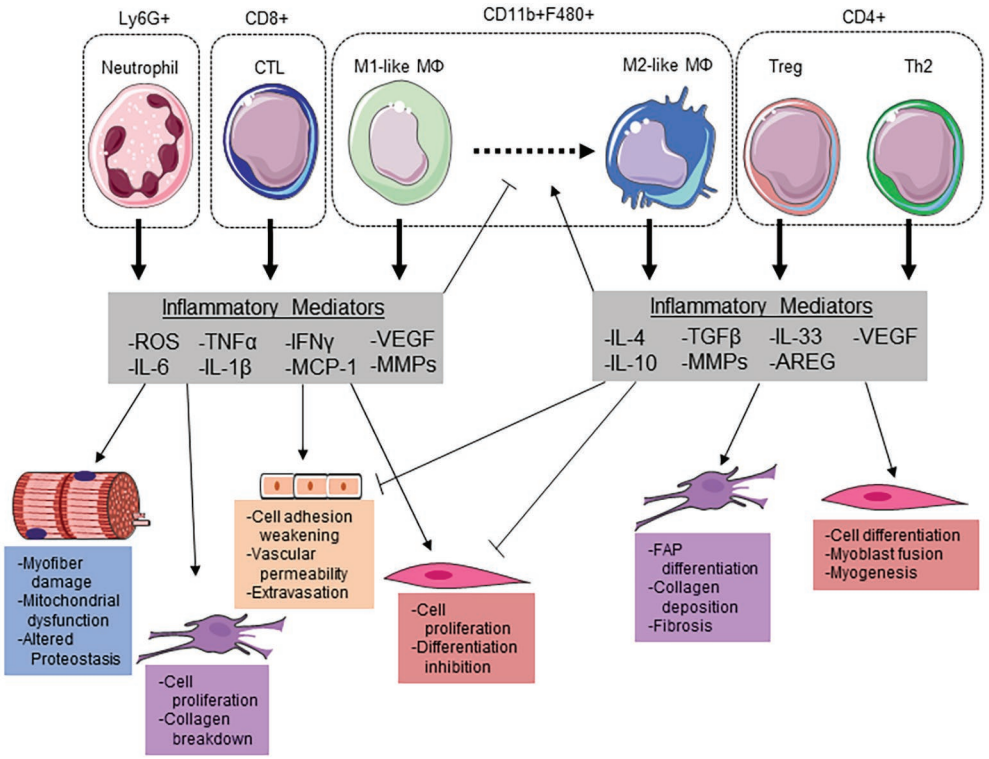

FIGURE 2 | Immune cells modulate the myofiber microenvironment through releasing key inflammatory mediators. Neutrophils potentiate cellular damage through the release of reactive oxygen species (ROS), which can induce myofiber damage and increase vascular permeability for extravasation. Additionally, neutrophils release pro-inflammatory cytokines interleukin (IL)-6, IL-1β, and tumor necrosis factor α (TNF-α) to stimulate satellite cell proliferation and suppress differentiation. IL-6 and ROS have been demonstrated to induce mitochondrial dysfunction/damage and altered proteostasis within the myofiber. Along with cytotoxic T-lymphocytes (CTLs), neutrophils release monocyte chemoattractant protein (MCP) 1 to recruit inflammatory (M1-like) macrophages (MΦ). Both infiltrating monocytes and resident MΦs can polarize to an M1-like phenotype to aid in phagocytizing damaged myofibrillar proteins and the breakdown of collagen in the ECM through matrix metalloproteinases (MMPs). Along with an increase in Interleukin-4 (IL-4), regulatory T-cells (Tregs) can release IL-33 to stimulate amphiregulin (AREG) to promote MΦ polarization to a M2-like phenotype. These MΦs then release transforming growth factor (TGF) β and IL-10 to promote fibro-adipogenic progenitor (FAP) cell differentiation, ECM remodeling, and suppress the pro-inflammatory milieu. The reduction in pro-inflammatory cytokines allows for satellite cell differentiation and myogenesis. This figure was made with Servier Medical Art (http://www.servier.com/Powerpoint-image-bank).

The ECM surrounding each muscle fiber can contain an assortment of fibroblasts, endothelial cells, immune cells, and satellite cells which are located beneath the basal lamina (Forcina et al., 2019). These cells have individual and coordinated roles in the response to local inflammation that accompanies muscle remodeling to damage, increased use/activity, and overload (Morgan and Partridge, 2020). Furthermore, changes within the ECM serve to initiate intracellular myofiber signaling through receptors on the sarcolemma in response to damage and mechanical stretch (Carson and Wei, 2000). The ECM is a rich source of growth factors and cytokines that can be released to regulate myofiber proteostasis and metabolic function (Forcina et al., 2019). Muscle fibers interact with the fibrous elements of the matrix through integrin receptors to sense mechanical signaling, and shifts in the expression of fibrous matrix proteins such as collagens have potential to alter intracellular signaling in muscle fibers (Carson and Wei, 2000). Thus, the cancer systemic environment has the potential to dysregulate myofiber gene expression and metabolic signaling through alterations to the ECM. Muscle fibrosis and dysregulation of the ECM has been reported in cachectic skeletal muscle from pancreatic cancer patients (Judge et al., 2018; Nosacka et al., 2020), and fibrosis can negatively impact myofiber growth and healing from damage (Bedair et al., 2007; Li et al., 2016a). Muscle inflammatory processes are established modulators of the ECM for healing and wound repair and also critical for muscle remodeling to exercise and overload. In fact, disruptions to IL-6 expression during overload-induced hypertrophy and during the recovery from disuse-induced muscle atrophy in mice can alter the hypertrophy response, collagen composition within the ECM, and muscle fibrosis in mice (White et al., 2009; Washington et al., 2011). Furthermore, during the initiation of compensatory overload-induced hypertrophy in mice an inflammatory and ECM gene expression is strongly induced (Carson et al., 2002). These observations demonstrate the complexity of the ECM response and local inflammation, since they drive hypertrophy with overload and healing from damage, but are also implicated in cancer-induced muscle wasting (Forcina et al., 2019). To this end, local inflammatory responses are subjected to precise temporal regulation and if this response

is altered muscle remodeling can be either attenuated or blocked (Howard et al., 2020), and further research is warranted to determine if cancer can disrupt the temporal aspects of local inflammation needed for successful remodeling. For example, inflammatory signaling can impact several cell types located in the muscle microenvironment leading to altered myofiber protein synthesis (Gao et al., 2017) and mitochondrial quality control (Gomez-Cabrera et al., 2016), which are known drivers of muscle wasting with cancer. In this section, three important cell types are highlighted that interact with the myofiber within the muscle microenvironment and their potential role in cancer and chemotherapy-induced muscle wasting is emphasized.

Satellite Cells

The role of the satellite cell in skeletal muscle physiological and biological responses to a variety of conditions, stimuli, and disease states has been extensively investigated since being first discovered by Mauro in 1961 (Mauro, 1961; Penna et al., 2010; He et al., 2013). The satellite cell is located between the myofiber sarcolemma and basal lamina and is not easily distinguished from a myonuclei with light microscopy. These cells remain in a quiescent state, but can be activated to proliferate and differentiate by a variety of growth factors and mitogenic stimuli (Bischoff, 1986a,b). Satellite cell differentiation can involve either fusion with existing myofibers to allow for myonuclear accretion or the formation of *de novo* myotubes. Therefore, the satellite cell has been extensively examined as a myogenic stem cell in skeletal muscle critical for muscle repair and growth processes. Both the regulation and disruption of satellite cell number, activation, proliferation potential, and differentiation have been widely investigated for 60 years (Forcina et al., 2019). Interestingly, one of the earliest described inducers of satellite cell proliferation was FGF, and this activation was also linked to damage disruption of the muscle ECM (Bischoff, 1986a,b). This line of inquiry coincides with investigation to this day involving satellite cell control by their microenvironment and the cells that are at the source of these mitogenic factors. There has been increasing interest in understanding the cancer environment's effect on satellite cell activity, which could directly impact cachectic muscle's diminished capacity for muscle regeneration (Schwarzkopf et al., 2006; Penna et al., 2010; He et al., 2013; Talbert and Guttridge, 2016). Deficits in the capacity for myogenesis, which is needed for successful muscle regeneration, have been reported in skeletal muscle from preclinical cachexia models and cancer patients, and involve an inability of satellite cells to effectively differentiate to complete the regenerative process (Penna et al., 2010; He et al., 2013). Due to the importance of the successful myogenic response in muscle regeneration, signaling pathways disrupting satellite cell activity have been actively investigated as potential therapeutic targets for cancer-induced muscle wasting (Schwarzkopf et al., 2006; He et al., 2013; Cerquone Perpetuini et al., 2018; Costamagna et al., 2020; Schmidt et al., 2020). To this end, skeletal muscle satellite cell differentiation has been reported to be rescued in colon-26 induced cachexia in mice through the inhibition or ERK signaling (Penna et al., 2010), and more recently IL-4 administration (Costamagna et al., 2020). Pax7 is a positive regulator of satellite cell proliferation, which has been reported to be disrupted in cachectic muscle from mice and cancer patients (He et al., 2013); dysregulation of Pax7 in cachectic muscle was driven by aberrant NF-κB signaling induced by circulating tumor derived factors. Wnt signaling is an established regulator of muscle satellite cell activity through the expression of myogenic regulatory factors and increased Wnt7a in colon-26 induced cachexia in mice can increase muscle regeneration by the improvement of muscle satellite regulation of differentiation and the induction of anabolic signaling through Akt/mTORC1 (Schmidt et al., 2020). Interestingly, mathematical modeling of colon-26 induced cachexia in mice recently predicted that blocking the myostatin/activin A pathway could attenuate cancer-induced muscle mass loss through the restoration of functional satellite cell differentiation (Farhang-Sardroodi and Wilkie, 2020). Depending on the extent of the damage, successful muscle regeneration including the initiation and resolution of local inflammatory processes involves infiltrating monocytes, resident MΦs, neutrophils, and T-cells (Morgan and Partridge, 2020). Local inflammatory responses are subjected to precise temporal regulation and if this response is altered muscle remodeling can be either attenuated or blocked (Howard et al., 2020), and further research is warranted to determine if cancer can disrupt the temporal aspects of local inflammation that contribute to disrupted satellite cell regulation of differentiation in cachectic muscle.

The satellite cell has been hypothesized to have critical roles in post-natal muscle growth, overload-induced hypertrophy, capacity of muscle regeneration after damage, and the development of sarcopenia. The development of modern molecular biology tools has shed light on the role of satellite depletion on these processes (McCarthy et al., 2011; Fry et al., 2014), but interesting gain of function experiments remain elusive. While the role of the satellite cell with some types of overload-induced growth and sarcopenia remains equivocal (Murach et al., 2018), the satellite cell has an established role in muscle regeneration from injury. Interestingly, there is emerging evidence that satellite cells have a regulatory role in ECM during overload-induced hypertrophy (Murach et al., 2018). Additionally, MΦs have been demonstrated to interact with satellite cells to regulate myogenesis (Dort et al., 2019). Pro-inflammatory monocytes and MΦs release cytokines, IL-6 and TNF-α, to stimulate satellite cell proliferation and inhibit differentiation until switching to an M2-like phenotype to promote differentiation and fusion (Arnold et al., 2007). T-cells have been demonstrated to maintain satellite cells myogenic potency *in vitro* (Fu et al., 2015), while T-cell deficient mice had reduced satellite cell proliferation contributing to blunted repair (Castiglioni et al., 2015; Deyhle and Hyldahl, 2018). There is significant interest in muscle microenvironment changes that occur with aging that could alter satellite cell regulation and serve to impede muscle regeneration from injury (Lee et al., 2019). Satellite cell number and activity are thought to be impacted by aging, and other regulators of satellite cell function such as the immune response are also aging targets (Forcina et al., 2019). Therefore, the impact of age on satellite cell activity could be a factor in the development and progression of cachexia due to the advanced age of many patients having

cancers that are associated with high cachexia incidence. These facts could directly impact our current mechanistic understanding of cancer cachexia, as preclinical modeling that account for the effects of sarcopenia and aging on the skeletal muscle microenvironment are needed to answer these critical questions.

Fibroblasts

Fibroblasts have a central role in the formation and remodeling of the skeletal muscle ECM which provides stability for the myofiber, blood vessels, and nerves (Chapman et al., 2016; Mendias, 2017). Fibroblasts aid in the synthesis and deposition of collagen, which predominate skeletal muscle connective tissues. Matrix metalloproteinases (MMPs) are largely responsible to the breakdown and deposition of collagen in the ECM. MMPs are released by immune cells, myofibers, satellite cells, epithelial cells, and fibroblasts themselves and are transcriptionally regulated by classic cytokine signaling cascades (Thomas et al., 1998; Liu et al., 2006; Phatharajaree et al., 2007; Yndestad et al., 2007; Iyer et al., 2016). These cells release MMPs to degrade several components of the cytoskeleton and stimulate *de novo* collagen deposition by the fibroblast (Sundararaj et al., 2009). Additionally, fibroblasts and satellite cells coordinate during skeletal muscle regeneration to limit fibrosis and promote healthy hypertrophic repair (Murphy et al., 2011).

The role of several immune cell types and inflammatory mediators in skeletal muscle fibroblast proliferation and collagen deposition has been extensively studied due to their integral functions in tissue repair (Van Linthout et al., 2014). In addition to providing structural support, fibroblasts are sensitive to mechanical stretch and maintain the ECM by aiding in the breakdown and synthesis/deposition of skeletal muscle collagens (Tomasek et al., 2002). Additionally, this mechanical sensitivity allows fibroblasts to release their own cytokines/chemokines for early neutrophil recruitment to aid in the breakdown of the damaged ECM through the action of MMPs, collagenases, and elastases (Baici et al., 1982; Kim and Luster, 2015); however, this mechanistic link in skeletal muscle requires additional work. On the other hand, innate immune cells can induce re-expression of fetal genes, activation of MMPs, fibro-adipogenic progenitor (FAP) differentiation, proliferation of fibroblasts, and myofibroblast formation (Tomasek et al., 2002; Gentek and Hoeffel, 2017; Tidball, 2017). Pro-inflammatory cytokines TNF-α, IL-6, and IL-1β are produced and secreted by Th cells and M1-like MΦs to stimulate fibroblast proliferation, while the anti-inflammatory TGFβ and IL-10 are predominately produced by Tregs and M2-like MΦs to stimulate fibroblast collagen synthesis and deposition (Van Linthout et al., 2014; Jablonski et al., 2015). M2-like MΦ release of arginase 1 (Arg1) also can stimulate fibroblast proliferation through the production of polyamines from arginase metabolism (Tidball, 2017). While the proper coordination of fibroblast proliferation and collagen formation is necessary for healthy skeletal muscle repair and remodeling, both reduced or exacerbated fibroblast activity can result in fibrosis and reduced skeletal muscle plasticity and overall functional capacity (Chapman et al., 2016). Fibrosis during the progression of cancer cachexia has received significant attention due to its role in reduced skeletal muscle plasticity and the loss of strength disproportionate to mass (Judge et al., 2018; Ramage and Skipworth, 2018); however, evidence of impaired regeneration and increased fibrosis in cachectic skeletal muscle is limited to cancer type and severity of the disease (Nosacka et al., 2020).

Endothelial Cells

Endothelial cells are cells that are associated with blood vessels, which vascularize skeletal muscle. Intuitively, skeletal muscle vascularization plays central roles in nutrient transportation (e.g., glucose delivery and CO_2 removal) and global/systemic communication (e.g., hormones and cytokines/myokines). When necessary, the myofiber, resident fibroblasts, and/or resident immune cells secrete cytokines and pro-inflammatory mediators to activate nearby endothelial cells initiating circulating immune cell extravasation into the damaged tissue (Vestweber, 2015). As previously discussed, the influx of neutrophils, activated monocytes, and pro-inflammatory MΦs are central for skeletal muscle repair and remodeling. A key function of neutrophils in promoting cell death is the generation of ROS through NADPH oxygenase-dependent mechanisms (Kolaczkowska and Kubes, 2013). While ROS can be destructive to skeletal muscle in several ways, ROS is an important regulator of skeletal muscle vascular permeability (Korthuis et al., 1985). VEGF has received significant attention for its role in stimulating angiogenesis and promotion of vascular permeability is vital for skeletal muscle repair and remodeling (Arsic et al., 2004). Endothelial cells rely heavily on self VEGF production and autocrine signaling for vascular homeostasis and this autocrine signaling cascade differs from exogenous or paracrine VEGF signaling (Lee et al., 2007). Interestingly, VEGF has been implicated as a regulator of DC differentiation and MΦ polarization (Li et al., 2016b). While monocytes and MCP-1 stimulate the release of VEGF by endothelial cells to increase myofibrillar vascularization during the later stages of repair (Hong et al., 2005), tumor associated macrophages (TAMs) promote the release of VEGF to promote tumor vascularization (Wynn et al., 2013). However, IL-4 inhibits basic Fibroblast Growth Factor (bFGF) induced angiogenesis (Volpert et al., 1998). Therefore, it is vital to understand off-target effects of modulating immune cells and their inflammatory mediators on the endothelium within the skeletal muscle microenvironment.

Endothelial cells have sustained significant attention in tumor cell biology given the importance of tumor vascularization in tumor cell growth and metastasis (Dudley, 2012; Li et al., 2016b). Naturally, treatments targeting endothelial cells and mitigating vascularization has emerged as a promising therapy; however, given the importance of vascular homeostasis in maintaining healthy tissue, the off-target implications of these treatments invoke caution (Morabito et al., 2006). While the effects of cachexia and chemotherapy on the skeletal muscle endothelium or the relationship between immune cells and the skeletal muscle endothelium has not been directly studied, we can glean information from studies examining aging, non-cancer-induced cachexia, and established inflammatory signaling and nutrient availability mechanisms. For instance, COPD patients suffering from cachexia have reduced skeletal

muscle capillary density partly caused by deconditioning (Jobin et al., 1998); given that cancer patients experience fatigue and reduced activity, we can expect that cachectic skeletal muscle with cancer has decreased vascularization. Reduced vascularization would diminish skeletal muscle's regenerative capacity resulting in increased fibrosis, reduced strength, and fatigue (Arsic et al., 2004; Allen et al., 2008; Inaba et al., 2018; Judge et al., 2018; Vanderveen et al., 2018). Additionally, aging processes contributing to frailty has been associated with endothelial dysfunction (Argiles et al., 2016). Inhibition of angiogenesis is a critical target for some chemotherapeutics aimed at preventing vascularization of solid tumors through suppressing VEGF (Zhang et al., 2002; Shih and Lindley, 2006; Ooyama et al., 2008; Bozzetti, 2020). The impact on non-cancerous tissues continues to be unearthed; however, endothelial dysfunction has been demonstrated during chemotherapy driven cardiotoxicity (Brown et al., 2012). Additionally, anti-angiogenic therapies should aim to achieve vascular normalization in order to maintain the delivery and anti-tumor efficacy of chemotherapeutics as opposed to eliminating tumor vascularity completely (Viallard and Larrivee, 2017). Chemotherapy's effect on skeletal muscle's endothelium has not been directly investigated. Given the neutrophil's role in ROS generation, the neutropenia associated with chemotherapy may inhibit the skeletal muscle's ability to increase vascular permeability and immune cell extravasation. Further work is needed to understand the impact of cancer and its treatments on skeletal muscle's vascular endothelium.

Other Cells Within the Skeletal Muscle Microenvironment: The Motor Unit

The nervous and musculoskeletal systems are inextricably linked through the motor unit and denervation, or the loss of the neuromuscular junction (NMJ), results in loss of function and rapid muscle atrophy (Bonaldo and Sandri, 2013). Denervation results in the affected myofibers losing their contractile capacity, which is then followed by a marked reduction in myofibrillar cross sectional area. If the NMJ is not restored, the affected myofibers will become unrecognizable, as they lose their sarcomeric structure and become fibrotic (Carlson, 2014). Similar to other atrophic conditions, MΦs have been identified as contributors to denervation-induced fibrosis (Mochizuki et al., 2005). Interestingly, denervation with cancer cachexia has been suggested in C26 mice (Daou et al., 2020), and neurotoxicity was observed with chemotherapeutic Folfiri (Barreto et al., 2016a); however, gastrointestinal cancer patients with cachexia had no significant loss of NMJs compared to weight stable or health controls (Boehm et al., 2020). Chemotherapeutic DOX disrupted the NMJ in sedentary rats while exercise was able to improve neuromuscular gene and protein expression (Huertas et al., 2020).

CONCLUSIONS AND FUTURE DIRECTIONS

The cellular environment surrounding the myofiber, or the ECM, is a mixture of mononucleated satellite cells, fibroblasts, endothelial cells, and immune cells. Together these cells cooperate and aid in locomotion (e.g., tendon formation/strength), systemic and local metabolic homeostasis, structural support, repair/remodeling, and systemic communication (e.g., myokines). Coordination of this complex network relies on each cell functioning to communicate within endocrine, paracrine, and autocrine mechanisms given a multitude of stimuli (Meng et al., 2015; Bersini et al., 2018). The complex nature of the skeletal muscle microenvironment invites difficulty in identifying key regulators of muscle mass and function loss with cancer and chemotherapy; however, targeting inflammation and immune cells prior to- or during cachexia's progression could promote widespread benefits given their regulatory roles in several skeletal muscle processes. While several inflammatory mediators (IL-6, TNF-α, TWEAK, TRAF6, INF-γ, and LIF) have been implicated as drivers of cancer-associated wasting, very little is known about their origin, whether skeletal muscle, immune cell, or tumor-derived (Flint et al., 2016; Jackman et al., 2017). In this review, we highlighted that resident and circulating immune cells can modulate several aspects of the skeletal muscle microenvironment through a multitude of mechanisms. To this end, omitting investigation of multiple cell types within the muscle's microenvironment during cachexia has the potential to hamper therapeutic developments/advancements leading to the current treatments, which have been unable to improve muscle function. Additional mechanistic inquiry is needed to understand how immune cells contribute to cancer and chemotherapy associated muscle mass and function loss.

For decades now, endurance and resistance exercise have been proposed as promising therapies as they broadly impact chronic inflammation, metabolic homeostasis, and muscle protein turnover (Al-Majid and McCarthy, 2001; Montalvo et al., 2018). There are barriers to exercise as a therapeutic for cancer cachexia as it is unlikely that a level of exercise to induce muscle mass can be performed by patients with debilitating conditions like cancer. However, muscle contractions induced by neuromuscular electrical stimulation was recently shown to improve muscle mass in breast cancer patients receiving chemotherapy (Guigni et al., 2019; Toth et al., 2020). Both resistance (Bredahl et al., 2020) and endurance (Smuder, 2019; Huertas et al., 2020; Montalvo et al., 2020) exercise have shown promise in mitigating chemotherapy-induced skeletal muscle toxicities in preclinical rats. Additionally, resistance (Hardee et al., 2016, 2018) and endurance (Puppa et al., 2012; Vanderveen et al., 2020) exercise can improve muscle mass and function in cachectic mice. Exercise also showed promise in preventing the loss of muscle mass and strength in C26 mice given 5FU + Oxaliplatin (Ballaro et al., 2019). Exercise oncology continues to be an active and intriguing field of study aimed at improving cancer patient's survival and quality of life (Schmitz et al., 2019). While traditional nutritional support has been unsuccessful, several nutraceuticals and nutritional anti-inflammatories have promise in mitigating cancer-induced wasting (Narsale et al., 2016). Moreover, there is growing interest in identifying plant-derived compounds to treat cachexia given that they can modulate multiple pathways, are inexpensive, and have low toxicity as prolonged treatments (Saklani and Kutty, 2008). Plant-derived compounds have been shown to regulate inflammation through the modulation of MΦ polarization with

cancer (Jia et al., 2014; Hu et al., 2017); however, the therapeutic efficacy of modulating MΦ phenotype and function during cancer-associated wasting has not been investigated.

AUTHOR CONTRIBUTIONS

JC planned, wrote, and edited manuscript. BV planned, wrote, and edited manuscript, created figures. EM edited and wrote manuscript. All authors contributed to the article and approved the submitted version.

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frontiers in Physiology

MINI REVIEW
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Muscle-Organ Crosstalk: Focus on Immunometabolism

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Skeletal muscle secretes several hundred myokines that facilitate communication from muscle to other organs, such as, adipose tissue, pancreas, liver, gut, and brain. The biological roles of myokines include effects on e.g., memory and learning, as well as glucose and lipid metabolism. The present minireview focuses on recent developments showing that exercise-induced myokines are involved in immunometabolism of importance for the control of e.g., tumor growth and chronic inflammation. In this review, immunometabolism is discussed as the non-immune related pathologies leading to an immune response and some degree of inflammation, which promotes metabolic abnormalities.

Keywords: metabolism, cytokines, exercise, physical activity, diabetes, cancer

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INTRODUCTION

The term "immunometabolism" was introduced as the interplay between metabolic and immunologic processes (Mathis and Shoelson, 2011). Immunometabolism refers to two concepts. One is how leukocyte and lymphocyte function is regulated by their internal metabolism. The other is how pathologies considered to be non-immune – such as obesity – result in an activation of the immune system, which promotes metabolic abnormalities increasing the risk of type 2 diabetes, cardiovascular diseases and cancer (Mathis and Shoelson, 2011). In this review, the main focus is on the latter understanding of the immunometabolism concept, and on how muscle activation through exercise can counteract some of the inflammatory processes related to these diseases.

Myokines are involved in mediating the multiple physiological, metabolic and immunological effects of physical activity (Pedersen et al., 2003a; Pedersen and Febbraio, 2012).

A single bout of exercise provokes an increase in systemic levels of IL-6 (Pedersen, 2013). In relation to exercise, IL-6 is released as a myokine from muscle into the circulation, and IL-6 plasma levels increase exponentially with exercise duration. Recent findings consolidate the pleiotropic nature of IL-6 and demonstrate a physiological role of this myokine in regulating clinically relevant parameters related to energy homeostasis and immune cell regulation in cancer (Severinsen and Pedersen, 2020).

Following the identification of muscle-derived IL-6, it has become evident that skeletal muscle cells are able to secrete more than 650 myokines (Khan and Ghafoor, 2019). The role of myokines has previously been reviewed (Pedersen et al., 2007; Pedersen, 2009, 2011, 2019; Walsh, 2009; Brandt and Pedersen, 2010; Arnold et al., 2011; Hamrick, 2011; Trayhurn et al., 2011; Pedersen and Febbraio, 2012; Raschke and Eckel, 2013; Pal et al., 2014; Ahima and Park, 2015; Benatti and Pedersen, 2015; Indrakusuma et al., 2015; Schnyder and Handschin, 2015; Whitham and Febbraio, 2016; Hoffmann and Weigert, 2017; Rodriguez et al., 2017; Ruiz-Casado et al., 2017; Diaz et al., 2018; Fiuza-Luces et al., 2018; Coelho-Junior et al., 2019; Das et al., 2019; Eckel, 2019; Garneau and Aguer, 2019;

Graf and Ferrari, 2019; Lee and Jun, 2019). Until now the biological function has been described for only approximately 5% of all known myokines. Nonetheless, the identification of the myokinome has provided a new paradigm and a conceptual basis for understanding by which mechanisms muscles communicate with other organs. Several of these myokines relate to immune function and inflammation. Low-grade inflammation is associated with several types of obesity-related diseases such as diabetes, cardiovascular disease, cirrhosis, and cancer. We suggest that control of this pathology-related inflammation can in part be ascribed by the release of immunogenic myokines. These are highlighted in **Figure 1**.

MUSCLE-IMMUNE-INFLAMMATION CROSSTALK

Muscle has impact on lymphocyte and neutrophil trafficking and inflammation (Duggal et al., 2019). During exercise, neutrophils as well as natural killer (NK) cells and other lymphocytes enter the blood. Exercise of high intensity and long duration leads to a decline in lymphocyte number, while the concentration of neutrophils increase (McCarthy and Dale, 1988; Pedersen and Hoffman-Goetz, 2000) via mechanisms that include adrenaline and cortisol. IL-6 has been shown to be involved in mediating the increase in cortisol (Steensberg et al., 2003).

Lack of exercise and obesity are accompanied by low level chronic inflammation (Pedersen et al., 2003b; Petersen and Pedersen, 2005; Pedersen, 2006; Pedersen, 2006, 2017; Benatti and Pedersen, 2015; Knudsen and Pedersen, 2015; Karstoft and Pedersen, 2016). The anti-inflammatory effects of exercise training are induced with each single bout of exercise as well as via training adaptation leading to a decrease in the amount of abdominal fat.

IL-6 increases with exercise and promotes the occurring of two cytokines with anti-inflammatory effects (Steensberg et al., 2003). IL-1 receptor antagonist (IL-1ra) blocks IL-1β signaling (Dinarello, 1994) and IL-10 prevents TNF-α production (Opp et al., 1995). A study in healthy humans showed that a bout of exercise or administration of IL-6 before infusion of endotoxin abolished the increase in plasma levels of TNF-α that was seen in a control situation (Starkie et al., 2003). It was concluded that a single bout of exercise mediates an anti-inflammatory signal, which is likely to be partly mediated by IL-6 (Pedersen, 2017).

Exercise can also induce anti-inflammatory effects via a reduction in abdominal fat (Rosenkilde et al., 2016). Abdominal adiposity, reflecting a high amount of visceral fat, is associated with cardiovascular disease, type 2 diabetes, dementia and all-cause mortality (Pedersen, 2009). Accumulation of visceral fat represents an important source of origin of chronic systemic inflammation, as it has been shown to be more inflamed than subcutaneous fat, constituting an important source of inflammatory markers (Yudkin, 2007).

Physical inactivity leads to an increased amount of visceral fat and consequently an environment of inflammation, which provokes a network of chronic diseases (Benatti and Pedersen, 2015).

Recent evidence exists that exercise training decreases the amount of visceral and cardiac fat mass (Christensen et al., 2019a,b; Wedell-Neergaard et al., 2019) mediated by muscle-derived IL-6 (Wedell-Neergaard et al., 2019) as described below.

MUSCLE-ADIPOSE CROSSTALK

Exercise-induced IL-6 has significant effects on fat metabolism (Pedersen, 2013, 2018). *In vivo* studies in humans show that rhIL-6 enhances lipolysis and fat oxidation (van Hall et al., 2003; Petersen et al., 2005). Epidemiological studies demonstrate that an association exists between abdominal adiposity and low fitness (Wedell-Neergaard et al., 2018a,b). Intervention studies show that reduced number of daily steps provoke accumulation of visceral adipose tissue (Olsen et al., 2008; Benatti and Pedersen, 2015), whereas exercise training reduced abdominal adiposity (Ross et al., 2000; Nordby et al., 2012). In a recent study, abdominally obese humans were randomized to tocilizumab (IL-6 receptor antibody) or placebo during an intervention of 12-weeks with either aerobic exercise or no exercise (Christensen J. F. et al., 2018; Wedell-Neergaard et al., 2019). While exercise training led to a reduction in visceral adipose tissue mass, this effect was completely abolished by IL-6 receptor blockade (Wedell-Neergaard et al., 2019).

It has also been hypothesized that exercise may induce browning of white adipose tissue (Rodriguez et al., 2017; Eckel, 2019; Townsend and Wright, 2019). Myokines with browning properties include irisin (Bostrom et al., 2012), meteorin-like (Rao et al., 2014), and IL-6 (Knudsen et al., 2014). The finding that exercise-induced myokines may induce browning of white adipose tissue has been demonstrated in rodent models, but not consistently so in humans (Norheim et al., 2014; Vosselman et al., 2015; Severinsen et al., 2020).

MUSCLE-CANCER CROSSTALK

Metabolic syndrome has been tied to risk of several types of cancer (Esposito et al., 2012). The vast amount of epidemiological studies demonstrate that exercise training decreases the risk of cancer and contributes to control disease progression. Exercise has also beneficial impact on anti-cancer therapy and improves physical and mental health in general. Being physically active reduces the risk of approximately 13 different cancer types (Moore et al., 2016; Pedersen et al., 2016; Christensen R. H. et al., 2018; Hojman et al., 2018). Exercise training after a diagnosis of breast cancer, prostate cancer and colorectal cancer are associated with an increased survival rate (Pedersen, 2018).

Given that cancer is associated with low level chronic inflammation, which may contribute to tumor progression, it is possible that the ability of physical training to create an anti-inflammatory environment, may facilitate exercise-induced protection on cancer growth (Hojman et al., 2018).

Pernille Hojman studied exercise effects on tumor growth in rodent models (Pedersen et al., 2016). She established a B16F10 melanoma model and exposed tumor-bearing mice to

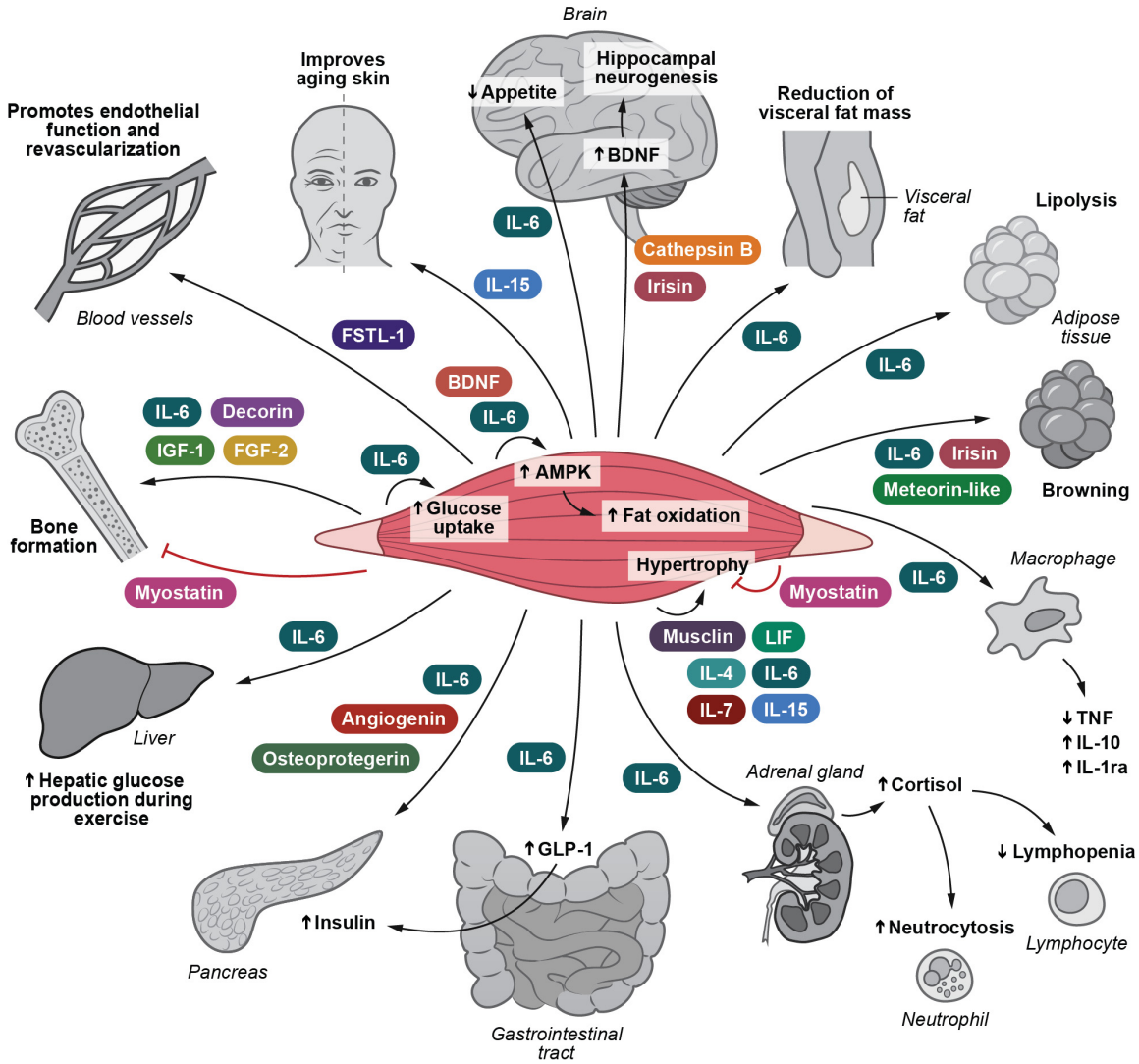

FIGURE 1 | I Irisin and Cathepsin B enhance BDNF production and thereby hippocampal neurogenesis. IL-6 inhibits appetite and stimulates lipolysis. IL-6 also plays a role in decreasing the amount of visceral fat. IL-6, irisin and meteorin-like are involved in turning white adipose tissue into a brown phenotype. IL-15 retards skin aging. IL-6, decorin, FGF-2 and IGF-1 positively influence bone formation. Myostatin negatively influence bone formation. Musclin, LIF, IL-4, IL-6, IL-7, and IL-15 are involved in mediating muscle hypertrophy, whereas myostatin obstructs muscle hypertrophy. IL-6 and BDNF stimulate AMPK activation and hence fat oxidation. IL-6 e stimulates glucose uptake and hepatic glucose output during exercise. IL-6 induces the expression of GLP-1 by the L cells of the intestine leading to enhanced insulin secretion. IL-6 exerts anti-inflammatory effects by inhibiting TNF production and by stimulating IL-1ra and IL-10 production. IL-6 enhances cortisol production, leading to neutrocytosis and lymphopenia. FSTL-1 has beneficial effects on endothelial function and revascularization of atherosclerotic blood vessels. Osteoprotegerin, angiogenin, and IL-6 possess beta-cell protective actions against inflammatory cytokines. AMPK, 5'-AMP-activated protein kinase; BDNF, brain-derived neurotrophic factor; FGF-2, fibroblast growth factor 2; FGF-21, fibroblast growth factor 21; FSTL-1, follistatin-related protein 1; GLP-1, glucagon-like peptide 1; IGF-1, insulin-like growth factor I; IL-1ra, IL-1 receptor antagonist; LIF, leukemia inhibitory factor; TGF-β, transforming growth factor β; TNF, tumor necrosis factor. Adapted with permission from Severinsen and Pedersen (2020).

wheel running or control. It appeared that exercising mice had a significant decrease in tumor mass and incidence.

Myokines are involved in mediating the effect of exercise on tumor growth. When breast cancer cells were treated with irisin, they were more likely to undergo apoptosis (Hojman et al., 2018).

The myokine oncostatin M (Pedersen et al., 2016) was shown to inhibit breast cancer cell proliferation. The myokine, secreted protein acidic and rich in cysteine (SPARC) was shown to reduce tumor in the colon of exercising mice (Aoi et al., 2013).

Exercise also induces acute increases in epinephrine and norepinephrine, which are involved in recruiting NK cells in humans during exercise. Breast cancer cells exposed to serum collected after a single bout of acute exercise and thereafter injected into mice, led to a reduction of tumor formation (Dethlefsen et al., 2017). This effect was, however, completely blunted when we blocked β-adrenergic signaling, the pathway through which epinephrine and norepinephrine work (Dethlefsen et al., 2017).

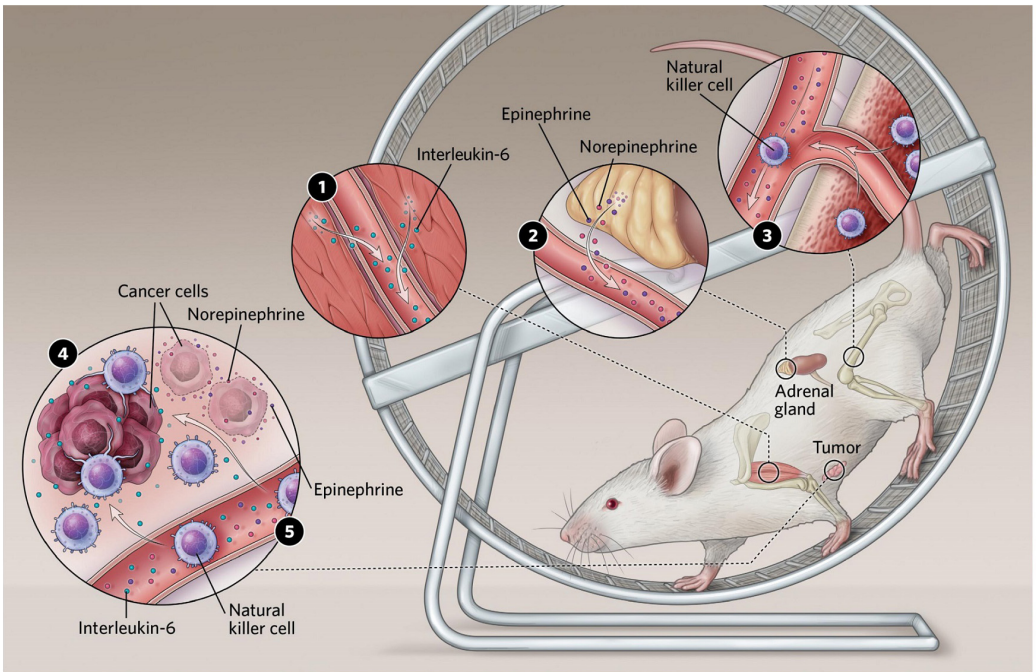

FIGURE 2 | (1) Exercising muscles release multiple compounds known as myokines. Several of these have been shown to affect cancer cell proliferation in culture, and some, including interleukin-6, slow tumor growth in mice. **(2)** Exercise stimulates an increase in levels of the stress hormones epinephrine and norepinephrine, which can both act directly on tumors and stimulate immune cells to enter the bloodstream. **(3)** Epinephrine also stimulates natural killer cells to enter circulation. **(4)** In mice, interleukin-6 appears to direct natural killer cells to home in on tumors. Reprinted with permission from Pedersen (2020). Illustrator: Scott Leighton. **(5)** Epinephrine and norepinephrine along with some myokines can inhibit tumor growth.

These findings suggested that epinephrine and norepinephrine play a key role in the cancer-inhibiting effects of exercise. To this end, catecholamine release has been linked to the best-characterized myokine, IL-6, which increases exponentially during exercise in humans. Muscle cells from rats have been shown to release IL-6 upon stimulation with epinephrine (Frost et al., 2004), and injection of a high dose of IL-6 in human subjects resulted in increased epinephrine levels (van Hall et al., 2003).

In the cancer-setting, Pernille Hojman and her team found that the inhibitory effects of exercise on tumor growth were mediated via a direct regulation of natural killer (NK) cells, where these were mobilized to the circulation and redistributed to the tumor tissue by a mechanism involving both epinephrine and IL-6. Blocking IL-6 signaling during exercise abolished the exercise-induced inhibition of tumor growth, suggesting that IL-6 plays a role in mediating anti-cancer effects (Aoi et al., 2013; Hojman et al., 2011; Hojman et al., 2018; Lucia and Ramirez, 2016; Manole et al., 2018; **Figure 2**).

In addition to the crucial increase of tumor-infiltrating NK cells with exercise, microarray analyses of the tumors revealed that 52% of the upregulated gene ontology pathways were linked to immunological and inflammatory responses, and qPCR analyses showed increased tumoral expression of several cytokines (Pedersen et al., 2016). Amongst these upregulated cytokines were Interferon-γ, which has been reported to stimulate immunoregulatory molecules on a wide selection of both healthy and diseased cells (Sun et al., 2018), and IL-15 known to stimulate activation and cytotoxicity of both NK cells and T cells (Guo et al., 2017). These clear associations between exercise and the immunogenic profiles of tumors makes it higly relevant to study the possible benefits of combining exercise with immunotherapy. These could either be checkpoint inhibitors or immune-stimulatory treatments.

MUSCLE-CARDIAC TISSUE CROSSTALK

The inflamed arterial wall is a hallmark in the development of cardiovascular disease. Given that each bout of exercise induces anti-inflammatory effects, mediated by IL-6, it is likely that transient increases in this myokine contributes to the protection against atherosclerotic disease.

Another myokine of importance for cardiac disease is follistatin-like 1 (FSTL1), which is expressed by skeletal as well as cardiac muscle cells (Shimano et al., 2012). FSTL1 promotes the function of endothelial cells and is involved in revascularization (Oshima et al., 2008; Ouchi et al., 2008), although its role in humans need to identified.

MUSCLE-LIVER CROSSTALK

Exercise stimulates an augmented production of glucose from the liver (Wasserman et al., 1991). In 1961, Goldstein (1961)

proposed that contracting muscle produced an exercise factor that could stimulate hepatic glucose output. Evidence exists that IL-6 plays a role in hepatic glucose output. This was the conclusion from a study in which young healthy males did 2 h of cycle ergometer exercise on 3 different days at: (1) a high intensity; (2) a low intensity; and (3) a low intensity + infusion of IL-6 at a concentration to mimic the systemic increase in IL-6 during exercise of high intensity. The results from this human experiment demonstrated that exercise-induced IL-6 is involved in triggering hepatic glucose output during exercise (Febbraio et al., 2004).

Muscle-Beta-Cell

Studying human primary muscle cell cultures established from triceps brachii, soleus and quadriceps identified two myokines, angiogenin and osteoprotegerin, which were shown to be triceps specific myokines, mediating anti-inflammatory actions and protecting beta-cell survival (Rutti et al., 2018). Moreover, it has been shown that IL-6 positively regulates β-cell mass *in vivo* (Ellingsgaard et al., 2008). The increase in IL-6 with each bout of exercise may be involved in protecting pancreatic β-cell mass and function.

OTHER MUSCLE-ORGAN CROSS-TALKS

Muscle-Brain Crosstalk

Regular exercise has beneficial effects on brain health (Cotman et al., 2007; Mattson, 2012). The fact that exercise is sensed by the brain suggests a direct crosstalk between working muscle and brain function (Pedersen and Febbraio, 2012; Benatti and Pedersen, 2015; Leardini-Tristao et al., 2019; Pedersen, 2019). Studies in humans (Erickson et al., 2011) and rodents (Kobilo et al., 2011) demonstrate a positive effect of exercise on hippocampus volume (Kobilo et al., 2011). In humans, brain-derived Neurotic factor (BDNF) Studies in humans demonstrate that BDNF is released from the brain in relation to exercise (Rasmussen et al., 2009; Seifert et al., 2010) and regular exercise for 3 months leads to an increase in the volume of hippocampus (Pajonk et al., 2010). In rodents, BDNF mRNA and protein increase in response to exercise (Pedersen and Febbraio, 2012; Benatti and Pedersen, 2015; Leardini-Tristao et al., 2019; Pedersen, 2019) and stimulate hippocampus growth (Loprinzi and Frith, 2019) as well as memory and learning (Vaynman et al., 2004a,b). Interesting studies in mice show that the myokines cathepsin-B (Moon et al., 2016) and irisin (Wrann et al., 2013) may be released from muscle to blood during exercise, passing from the blood to the brain and directly provoking an increase in brain BDNF. When IL-6 is centrally applied in mice, it suppresses feeding (Timper et al., 2017). Moreover, a much higher IL-6 concentration applied peripherally reduces the intake of food, suggesting that high systemic IL-6 concentrations may pass from the blood to the brain and regulate appetite. The latter results indicate that IL-6 released from muscle during exercise of high intensity and long duration (Febbraio and Pedersen, 2002), may lead to a decrease in appetite.

Muscle-Muscle

Some myokines can exert their effects on the muscle itself. One of these is IL-6, which can work in both an endocrine and a paracrine manner within the muscle (Pedersen and Febbraio, 2008, 2012). In a metabolic perspective, studies in humans show that IL-6 is capable of increasing glucose uptake by a mechanism that involves activation of AMPK (Carey et al., 2006). Moreover, IL-6 increases insulin-stimulated glucose uptake *in vitro* as well as in *in vivo* in health humans (Carey et al., 2006). Furthermore, IL-6 increases fatty acid oxidation via AMPK activation (Kahn et al., 2005; Carey et al., 2006). BDNF is yet another myokine, which stimulates AMPK activation and thereby lipid oxidation. BDNF works in an autocrine or paracrine manner (Matthews et al., 2009). Finally, Musclin is an exercise-induced factor (Nishizawa et al., 2004) that promotes mitochondrial biogenesis in murine muscle (Subbotina et al., 2015).

Muscle-Gut

IL-6 stimulates glucagon-like peptide-1 (GLP-1) secretion in mice from both pancreatic β-cells and intestinal L-cells, thereby enhancing insulin secretion. A recent human study from our group (Lang Lehrskov et al., 2018) demonstrates that IL-6 slows down the rate of gastric emptying. Thereby IL-6 indirectly exerts beneficial effects on postprandial glucose (Woerle et al., 2008).

Muscle-Skin

Studies in exercising mice and humans suggest that muscle-derived IL-15 contributes to avoid aging of the skin (Crane et al., 2015). The latter study showed that that exercise regulates muscular IL-15 expression via skeletal muscle AMPK.

THE POTENTIAL CLINICAL IMPACT OF MYOKINES IN IMMUNOMETABOLISM

Myokines have been identified which include effects on e.g., lipid and glucose metabolism, browning of white fat, beta-cell-function, endothelial cell function and tumor growth. The biological and physiological identification of several myokines has identified these to be useful biomarkers for monitoring the exercise training, which is necessary in order to apply exercise as medicine for patients with specific diseases, such as diabetes, cardiovascular diseases and cancer. The identification of new myokines, playing specific roles in immunometabolism, may lead to new therapeutic targets for lifestyle-related diseases.

CONCLUSION

During exercise, myokines play a role in regulating immune cell trafficking, inflammation and metabolism. Exercise training thereby represents a strategy to induce a anti-inflammation and improved metabolism, which may contribute to decrease the risk or progression of cancer and type 2 diabetes as well as other chronic disorders.

AUTHOR CONTRIBUTIONS

MB and BP wrote the manuscript. Both authors contributed to the article and approved the submitted version.

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REVIEW
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Crosstalk Between Innate and T Cell Adaptive Immunity With(in) the Muscle

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Growing evidence demonstrates a continuous interaction between the immune system and the skeletal muscle in inflammatory diseases of different pathogenetic origins, in dystrophic conditions such as Duchenne Muscular Dystrophy as well as during normal muscle regeneration. Although one component of the innate immunity, the macrophage, has been extensively studied both in disease conditions and during cell or gene therapy strategies aiming at restoring muscular functions, much less is known about dendritic cells and their primary immunological targets, the T lymphocytes. This review will focus on the dendritic cells and T lymphocytes (including effector and regulatory T-cells), emphasizing the potential cross talk between these cell types and their influence on the structure and function of skeletal muscle.

Keywords: myoblasts, macrophages, dendritic cells, effector T cells, regulatory T cells, polymyositis, Duchenne muscular dystrophy

INTRODUCTION

The Interactions between the immune system and the skeletal muscle in different pathological conditions such as inflammatory myopathies and muscular dystrophies represent a growing field of investigation. Although the macrophage, one important component of innate immunity has been extensively studied in different aspects of muscle regeneration, as well as adjuvants for cell or gene therapy (Chazaud, 2020), much less in known about DC and its partner, the T cell.

During muscle repair macrophages were shown to acquire first a pro-inflammatory and then an anti-inflammatory/regulatory profile, crucial for the maintenance and resolution of the inflammatory process.

It is also known that during muscle repair, the differentiation of muscle progenitors into mature muscle fibers involves a complex orchestration of different cell-cell and cell-matrix interactions as well as interaction with many soluble molecules secreted by these different components present within the regenerating muscle environment. Curiously, many of these mediators are molecules

shared with the immune system (Tacke et al., 2009; Costamagna et al., 2015; Sharma and Rudra, 2018). In fact, the cytokines produced by cells of the immune system are not immune-exclusive and many of them are essential for the homeostasis of different tissues. Healing processes in general, use immune mediators as messengers able to reorganize the tissue back into a homeostatic steady state (Tacke et al., 2009; Costamagna et al., 2015; Sharma and Rudra, 2018). However, the end point of the healing process is not always favorable, and fibrosis may occur, precluding healthy tissue from re-emerging. The differences in the possible outcome, healthy vs. fibrotic tissue is strongly dependent upon the correct spatial and temporal secretion of these cytokines.

In healthy muscle, innate immune cells, mostly macrophages, sit between the fibers, in the epimysium and perimysium. These macrophages can be viewed as scavengers for debris, but also as helpers, contributing to muscle formation/regeneration and homeostasis (Lichtman et al., 2016; Zhang et al., 2017; Smigiel and Parks, 2018). When any kind of injury hits a tissue, including muscle, neutrophils are the first cells to migrate in, attracted by chemokines, particularly IL-8, produced by the lesioned endothelium (Perobelli et al., 2015; Silva-Barbosa et al., 2015; Ley et al., 2018).

Very early after injury, resident macrophages and incoming neutrophils are activated by a number of inflammatory stimuli including Danger Associated Molecular Patterns (DAMPs) from pathogens or mammalian cells themselves. DAMPs can trigger a competent immune response (Matzinger, 1994; Matzinger, 2002) after binding to the cognate receptors, Pathogen Recognition Receptors (PRR) present on innate immune cells and on various other cell types (Medzhitov et al., 1997; Medzhitov and Janeway, 2002). The prototypical PRRs are the very conserved family of Toll Like Receptors (TLR),a transmembrane protein first known to regulate caudo-ventral orientation of drosophila embryogenesis. Interestingly, when the TOLL molecule is disrupted, it causes a morphological defect, but more importantly, the flies die of fungal infection indicating that TOLL is also involved in immunity (Lemaitre et al., 1996). The major cell types expressing TLRs are the antigen presenting cells: dendritic cells, macrophages and activated B cells. Yet, other cells of the immune system and others, including muscle, also carry them (Trinchieri and Sher, 2007; Tournadre et al., 2010). More than ten different types of TLRs have been described in mammals, recognizing molecules characteristic of different pathogens or of internal damage. The intracellular or cytoplasmic domain is conserved between TLRs and interleukin-1 family of receptors (IL-1R) and as consequence of activation, these receptors elicit a very potent inflammatory reaction in general (Lemaitre et al., 1996; Medzhitov et al., 1997; Medzhitov and Janeway, 2002).

In the case of an injured muscle, DAMPs derived from the necrotic fiber and endothelial cells, and include Histidyl-tRNA synthetase (HRS), High Mobility Group1 Binding protein (HMGB1) and extracellular ATP. The HMGB1 protein, for example, binds to its specific PRR, TLR4 (Sciorati et al., 2016). Macrophages and dendritic cells, which both express TLR4, are activated in such a way that they, not only produce a number of proinflammatory cytokines (TNF-α, IL-1, IFN-α, IFN-β), but also activate oxidative stress and nitric oxide production, becoming competent antigen presenting cells (Mogensen, 2009; Kawai and Akira, 2010; Cheng et al., 2015; Liu and Cao, 2016). This means that if they meet T lymphocytes, which can recognize the antigen on their surface complexed to MHC molecules, the T cells will become activated. As we will describe in this review, T lymphocytes together with macrophages and DCs, are all found in inflammatory muscle tissue, inflammatory myopathies such as myositis and genetically inherited degenerative diseases of the skeletal muscle, such as Duchenne muscular dystrophy (DMD) (Deyhle and Hyldahl, 2018; Sass et al., 2018). We will provide evidence that connects macrophages, DCs, T effector and T regulatory cells and their secreted molecules. The discussion includes, not only a description of those interactions, which are mediated by the major histocompatibility complex (MHC) and the T-cell receptor, but also cell-cell and cell-matrix interactions, in a molecular context of self-within-self recognition (de Sousa et al., 1991).

Macrophages: Role in Muscle Regeneration

Macrophages are professional phagocytic cells, since they have a high capacity to eliminate dead and apoptotic cells, cell debris as well as a large number of pathogens. In addition, they produce Reactive Oxygen Species (ROS), secrete soluble factors such as cytokines and chemokines; and present antigens to T lymphocytes. Moreover, macrophages participate in the maintenance of tissue homeostasis and develop specialized functions in a tissue dependent manner (Medzhitov, 2010; Varol et al., 2015). These cells have a remarkable plasticity to adapt to the milieu they are in. Accordingly, they can acquire a pro-inflammatory or an anti-inflammatory/regulatory profile, crucial for the maintenance and resolution of any inflammatory process. In short, inflammatory components such as LPS and cytokines like IL-12, IFN-γ, and TNF-α polarize monocytes and macrophages into a pro-inflammatory population (also known as M1 or classically activated macrophages). Conversely, immunocomplexes, glucocorticoids, and cytokines such as IL-4, IL-13, and IL-10 can induce subpopulation of anti-inflammatory macrophages (known as M2, alternatively activated or regulatory macrophages) (Italiani and Boraschi, 2014; Murray et al., 2014; Vannella and Wynn, 2017; Jurberg et al., 2018).

As previously described, muscle repair is characterized by inflammation (Tidball, 1995; Saclier et al., 2013). It has been shown that pro-inflammatory macrophages co-localize with proliferating myoblasts whereas macrophages expressing anti-inflammatory markers (appearing concomitantly during muscle regeneration) are preferentially associated with myogenin-positive differentiated myoblasts (Lepper et al., 2011). Although $Pax7^+$ satellite cells are the only muscle stem cell responsible for muscle regeneration (Warren et al., 2004; Murphy et al., 2011), efficient muscle regeneration has been shown to depend on signaling from other cell types, in particular macrophages. For example, $Ly6C^+CCR2^+$ monocytes differentiate into different

macrophages that directly support myogenesis, protect muscle precursor cells from death, and stimulate myoblast proliferation, differentiation, and fusion. Moreover, strategies that prevent the arrival of monocytes into the skeletal muscle after tissue injury result in impaired tissue repair (Cantini et al., 1994; Warren et al., 2005; Summan et al., 2006; Arnold et al., 2007; Contreras-Shannon et al., 2007; Martinez et al., 2010).

It has also been shown that co-cultures of macrophages with myoblasts or macrophage-derived conditioned medium, stimulate myoblast proliferation and delay their differentiation (Johnson and Allen, 1990; Merly et al., 1999; Arnold et al., 2007; Bencze et al., 2012). Furthermore, several pro-inflammatory mediators, including TNF-α, IL-1β, IL-6, HGF, and IGF-I, have been shown to promote myoblast proliferation (Sheehan and Allen, 1999; Li, 2003; Zhang et al., 2013; González et al., 2017; Chaweewannakorn et al., 2018). In particular, HGF has been shown to enhance the migration of human myoblast *in vitro*. This effect is potentiated by the presence of extracellular matrix (ECM) proteins, with the involvement of matrix metalloproteinases and the MAPK/ERK pathways (Zhang et al., 2013). Interestingly the direct contact between macrophages and myogenic cells protects myoblasts and myotubes from cell death; this cell-cell contact being mediated by cell adhesion interactions, including VCAM-1/VLA-4, ICAM-1/LFA-1, and CX3CL1/CX3CR1 (Sonnet et al., 2006; Lesault et al., 2012).

Overall, these data highlight the importance of macrophages as myoblast supporting cells in the regeneration processes (Chazaud et al., 2003; Briggs and Morgan, 2013).

Dendritic Cells and Muscle Inflammation

Dendritic cells (DCs) are at the interface between innate and adaptive immunity. They are professional antigen-presenting cells (APCs); being known as sentinels of the immune system. They are resident components in non-lymphoid and lymphoid tissues where they take up antigens, migrate into draining lymph nodes, and trigger antigen-specific T and B cell responses (Padilla and Reed, 2008; Hubert et al., 2019). Upon maturation, DCs up-regulate co-stimulatory molecules and MHC Class II molecules (HLA-DR) and secrete a variety of cytokines. Depending on the tissue, the pathogen and the microenvironment, DCs promote a specific and adequate immune response toward Th1, interferon-γ (IFN-γ) secreting T lymphocytes, or other T cell phenotypes like Th2, Th17, or even Treg lymphocytes. Moreover, DCs play a crucial role in activating $CD4^+$ and $CD8^+$ T cells, B lymphocytes (toward autoantibody production), as well as providing different patterns of cytokine secretion depending on the environment and stimulus they receive (Hubert et al., 2019).

Using a murine model of muscle regeneration, it was demonstrated that $MHCII^+$ DCs and macrophages are present within uninjured muscle, and after a transverse crush injury in both anterior tibialis muscle, those cells increased and remained high until day 6 (Pimorady-Esfahani et al., 1997). This data is in agreement with another study, also in a myoinjury model induced by injection of notexin into the anterior tibialis and paravertebral muscle in mice. The authors showed that after injury, the resident macrophages recruit neutrophils and monocytes from the blood, which are progressively substituted by inflammatory DC's in the regenerating muscle (Brigitte et al., 2010). Both macrophages and inflammatory DCs are important in the response to muscle injury by recognition of self-molecules (for example HMGB-1, SAA1, HSP, DNA, RNA) released from damage cells through TLRs (TLR2/4 and TLR7/8), thus triggering inflammation and tissue repair. However, in dystrophies and myositis, the overwhelming release of cytokine and over-active TLRs can lead to chronic and destructive inflammation (De Paepe, 2020). As such, DCs can be placed as relevant innate immune cells in the context of skeletal muscle inflammation.

Despite the suggested role in a regeneration model, where macrophages seem to have a more relevant and understood role, DCs seems to have a greater participation in Idiopathic Inflammatory Myositis (IIMS).

Idiopathic inflammatory myositis (IIMs) corresponds to a heterogeneous family of diseases with a chronic or subacute onset, involving immune cells and the injured tissue. More recently, IIMs have been divided into four more clearly defined clinical entities, namely dermatomyositis, inclusion body myositis, immune-mediated necrotizing myopathy, and anti-synthetase syndrome (Mariampillai et al., 2018). Over the past years, many studies have tried to characterize the role of the immune cells in IIMs (Tripoli et al., 2020). The evidence for IIMs being an immune-mediated disease comes from the presence of cellular infiltrates within the muscle biopsies, T cell-mediated myocytotoxicity, autoantibodies in the peripheral blood, and association with MHC class I overexpression (Syed and Tournadre, 2015). IIMs are characterized by high levels of circulating cytokines and chemokines, as well as by inflammatory cellular infiltrates, including macrophages, DCs, $CD8^+$ T cells (predominantly affecting the endomysium in polymyositis) and $CD4^+$ T cells, which affect the perimysium in dermatomyositis (Wiendl et al., 2005; Huang et al., 2018). Although the involvement of DCs has been reported in IIMs (Greenberg, 2007; Greenberg et al., 2007; Wirsdörfer et al., 2016; Hubert et al., 2019), their exact role has not yet been defined.

Two different types of dendritic cells have been described in the muscle infiltrates of IIM patients, with a predominance of plasmocytoid DCs in dermatomyositis, and monocyte-derived DCs in polymyositis and inclusion body myositis (Pimorady-Esfahani et al., 1997; Wienke et al., 2018).

Interestingly, we have observed that human LPS-activated monocyte derived DCs tightly interact with human myoblasts and myotubes. This interaction seems to trigger myoblast proliferation, migration, and cytokine release and to impair myotube differentiation, thus suggesting that activated DCs inhibit myotube formation and muscle regeneration. A similar effect was observed when myoblasts and myotubes were incubated with TNF-α, IFN-γ, and, TGF-β, suggesting a role of circulating cytokines, in addition to the requirement for cell-cell contact. Moreover, co-injection of human myoblasts and DCs into freeze-injured *tibialis anterior* muscle of immunodeficient mice enhanced human myoblast migration, although the absolute number of human muscle fibers was unchanged (Ladislau et al.,

2018), similar to what had been shown for macrophages (Bencze et al., 2012). Similarly, increased numbers of activated DCs are seen in inflamed muscle (Pimorady-Esfahani et al., 1997; Padilla and Reed, 2008; Tournadre and Miossec, 2008) suggesting that DCs may also present antigens to T cells at the site of the lesion during myositis, in addition to the classic antigen-presentation in the draining lymph nodes (Hughes et al., 2016). This could be the trigger for autoantibodies production in some types of IIMs. Interesting, myoblasts and muscle fibers from inflammatory myopathies do express molecules typically expressed by APC and/or T cells, namely ICAM-1, HLA-DR, HLA-ABC, CTLA-4, CD28, BB-1, and B7-H1 increasing the chances of having a positive loop on immune activation within the muscle, with modulation of T cell activation and its fate.

The direct participation of DCs in the pathophysiology of inflammatory myopathies was provided in a murine model of polymyositis in C57BL/6 mice, consisting of the transfer of bone marrow-derived dendritic cells (BMDC) pulsed with a skeletal muscle specific antigen (the HILIYSDV peptide, derived from skeletal muscle C protein fragment). Seven days after immunization, the animals presented muscle lesions, induced by DCs, like the features observed in polymyositis. Importantly, such injury was mediated by $CD8^+$ T cells since anti-CD8 (but not by anti-CD4) depleting antibodies suppressed disease progression. (Kohyama and Matsumoto, 1999; Okiyama et al., 2014, 2015).

Studies of DCs in Duchenne muscular dystrophy are much scarcer than those reported for myositis. However, some data point to an important role of DCs, since TLR7 expressed on DCs binds to RNA and triggers cytokine production, enhancing the inflammation/degeneration/regeneration cycle. Among the cytokines released, the transforming growth factor (TGF)-β seems to be strongly induced in symptomatic patients, which would explain the participation of DCs, and their consequent interactions with T cells, keeping a positive feedback loop toward the maintenance of a fibrotic and dysfunctional muscle (Mbongue et al., 2014; Rosenberg et al., 2015).

Lastly, it is worth mentioning that the research about DCs during regeneration, myositis and DMD is complicated due to the small number of these cells in the muscle and that their presence probably occurs at the beginning of the disease development. Since patients generally arrive at the hospital once the disease is already established, possibly the role of DC is not relevant at this late time point.

T Cells in Idiopathic Inflammatory Myopathies and Duchenne Muscular Dystrophy

As mentioned earlier, immune cellular infiltrates including T cells, DCs and macrophages are present in muscle biopsies of inflammatory muscle diseases (Syed and Tournadre, 2015). In this context, with regard to idiopathic inflammatory myositis, an important participation of $CD4^+$ Th1, and Th17 cells, B lymphocytes, $CD8^+$ T lymphocytes and type I interferon has been reported (Tournadre and Miossec, 2012; Moran and Mastaglia, 2014; Reed et al., 2015; Crowson et al., 2019; Patwardhan and Spencer, 2019). The mechanisms involved in the pathophysiology of the different IIMs seem to differ. While $CD8^+$ T cells seem to be important in the pathogenesis of polymyositis and inclusion body myositis, $CD4^+$ T cells and B cells play a predominant role in the pathogenesis of dermatomyositis (Rosenberg et al., 2015; Syed and Tournadre, 2015).

Also, the relevance of cytokines in the skeletal muscle lesions seems to be vary according to the IIMs. While type I interferon has been detected in the muscle fibers of patients with dermatomyositis, as well as in plasmacytoid dendritic cells and in the endothelial cells in capillaries, overexpression of IFN-γ induced genes has been associated with inclusion body myositis (Reed et al., 2015; Crowson et al., 2019; Patwardhan and Spencer, 2019).

In the endomysium of patients with inclusion body myositis, dermatomyositis and polymyositis, the presence of T lymphocytes expressing restricted TCR families, in particular Vα2 and Vβ3, suggests that clones capable of recognizing autoantigens participate in the pathophysiology of these diseases (Lindberg et al., 1994). Similarly, in patients with polymyositis, it was observed that endomysial $CD8^+$ T cells surround and invade muscle fibers that express MHC class I antigens, with the consequent release of cytotoxic molecules, tissue destruction and release of autoantigens (Hohlfeld and Engel, 1991; Lindberg et al., 1994; Kohyama and Matsumoto, 1999; Levine et al., 2007; Tournadre and Miossec, 2012; Mbongue et al., 2014; Moran and Mastaglia, 2014; Reed et al., 2015; Rosenberg et al., 2015; Syed and Tournadre, 2015; Patwardhan and Spencer, 2019; Crowson et al., 2019). Moreover, numerous $CD4^+$ and $CD8^+$ T lymphocytes with the phenotype of terminally differentiated cells have been observed in polymyositis and dermatomyositis patients (Crowson et al., 2019). Such cells revealed a cytotoxic capacity, expression of receptors related to NK cells being potent IFN-γ and TNF producers (Fasth et al., 2009). Moreover, *in vitro* studies have shown that such lymphocyte subpopulations are cytotoxic to myotubes (Loell et al., 2011).

Despite previous studies showing increased frequency of highly differentiated $CD8^+$ T cell effector memory and terminally differentiated effector cells in patients with dermatomyositis and polymyositis, a recent study evaluating patients with various inflammatory myopathies, as well as patients with immune-mediated necrotizing myopathy, reported the presence of such a subpopulation only in patients with inclusion body myositis (IBM) (Greenberg et al., 2019), which could justify the resistance of these patients to treatment with corticosteroids, since terminally differentiated effector cells seems to be resistant to corticotherapy (Benveniste and Allenbach, 2019).

In Inclusion Body myositis, it has been proposed that $CD8^+$ terminally differentiated memory effector (TEMRA) T cells are involved in the pathophysiology of the disease through mechanisms involving cytotoxic enzymes (perforin and granzyme) as well as being mediated by IFN-γ, leading to an increase in the expression of HLA class I molecules, endoplasmic reticulum stress and proteasome dysfunction, with a consequent induction of rimmed vacuole formation and degenerative features (Arahata and Engel, 1984).

More recently, some studies focusing on polymyositis and dermatomyositis (not including inclusion body myositis) have suggested the participation of Th17 cells and the cytokines IL-17, IL-22, and IL-6 in the pathophysiology of IIMs. Moreover, IL-17A has been reported in the muscle tissue of patients with IIMs and *in vitro* studies suggest that IL-17A could play a role in the pathophysiology through mechanisms involving chemokine upregulation, increased inflammation and decreased cell migration and myogenic differentiation. Although such findings may point to new therapeutic perspectives, some results remain controversial and further work is still needed (Tournadre and Miossec, 2012; Moran and Mastaglia, 2014; Syed and Tournadre, 2015).

Pioneer studies in DMD patients revealed that the intramuscular inflammatory infiltrate is mainly composed by T lymphocytes (especially $CD8^+$ T cells) and macrophages (Arahata and Engel, 1984). Since then, several studies have been carried out to clarify the participation of the immune system in the pathophysiology of this disease. In this respect, it has been proposed that the absence of dystrophin and subsequent muscle cell damage, would result in the release of intramuscular antigens that could be specifically recognized by cells of the immune system (Spencer and Tidball, 2001). In such a context, it has also been observed that T lymphocytes present in the muscle tissue of patients with DMD predominantly express TCR Vβ2, and that this is not a characteristic shared by diseases involving inflammation of muscle tissue, since it was not detected in patients with polymyositis (Arahata and Engel, 1984). In addition, most patients with DMD have a conserved sequence of four amino acids in the CDR3 region of the TCR Vβ2, suggesting that the T cells present in the inflammatory infiltrate may recognize a specific muscle antigen (Mantegazza et al., 1993; Gussoni et al., 1994). Moreover, it has also been demonstrated that all DMD muscle fibers that were invaded by $CD8^+$ T cells expressed MHC class I molecules on their surfaces (Emslie-Smith et al., 1989). Once activated, cytotoxic $CD8^+$ T lymphocytes could then migrate and recognize specific peptides on the surface of muscle fibers triggering the release of perforin, granzyme and TNF-α, resulting in tissue damage.

In a cohort of 75 DMD patients, we have observed that increased percentages of circulating $CD4^+CD49d^+$ and $CD8^+CD49d^+$ T lymphocytes were correlated with a more rapid progression of the disease. Functionally, T cells from the more severely affected patients exhibited higher trans-endothelial and fibronectin-driven migratory responses when compared to healthy individuals (Pinto-Mariz et al., 2015).

We also observed a higher expression of fibronectin in the muscle of DMD patients, especially those with a worse prognosis, that is, those who lost the ability to walk before the age of ten. More importantly, both $CD49^+CD4^+CD3^+$ and $CD49^+CD8^+CD3^+$ were detected within the fibronectin-containing network of the injured muscle (**Figure 1**). Considering the haptotactic role of fibronectin on T lymphocytes (further enhanced upon activation), the increased production of this protein in muscle tissue of dystrophic patients could enhance the recruitment of more activated T cells toward the lesion. In fact, $CD49d^+$ T cell subsets were found in muscular

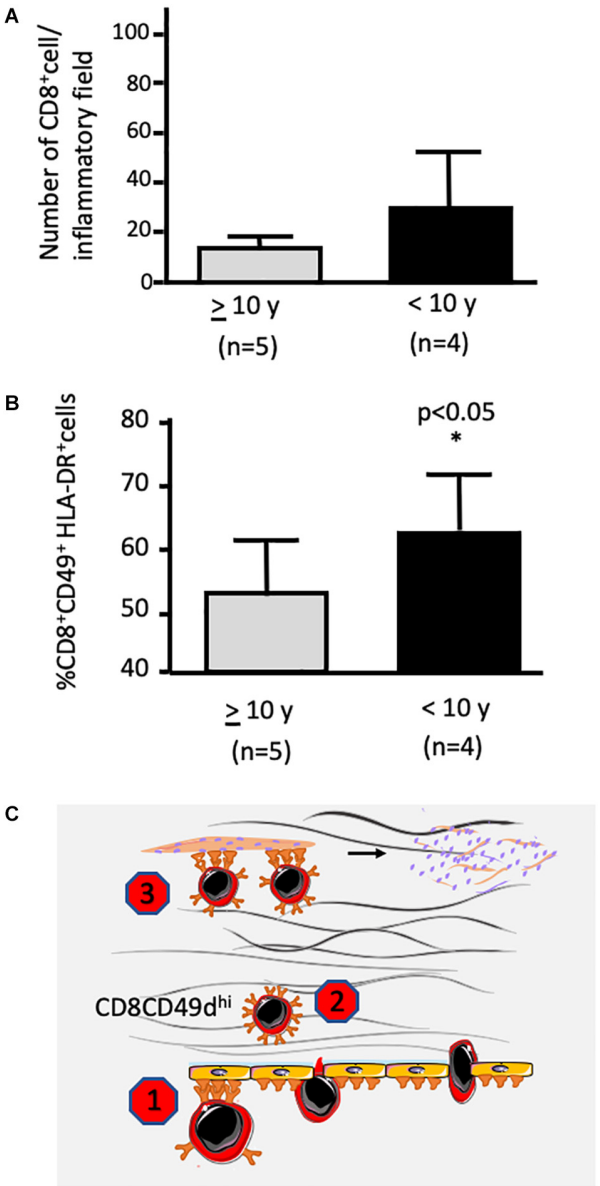

FIGURE 1 | Presence of infiltrating CD8+ T lymphocytes within the muscle of a patient being Duchenne muscular dystrophy: possible inhibition by VLA-4 inhibitors. **(A,B)** reveal $CD49d^+HLA\text{-}DR^+CD8^+$ T cells within the muscular tissue. The biopsies were performed at the onset of the disease. Patients were split according to loss of ambulation before or after 10 years of age. Data were calculated by counting labeled cells by *in situ* immunofluorescence made on frozen sections of muscle biopsies for simultaneous detection of CD8, HLA-DR, and CD49d. **(A)** depicts the numbers of $CD8^+$ cells per inflammatory field, whereas **(B)** reveals that the relative numbers of $CD49d^+HLA\text{-}DR^+CD8^+$ T were significantly higher in the muscles from children who lost ambulation before the age of 10 years (slightly modified from Pinto-Mariz et al., 2015). **(C)** illustrates the notion that CD49d inhibitors can block the VLA-4 activity on the transendothelial migration of T lymphocytes (Chazaud, 2020), as well as upon the intramuscular migration of the cells through a fibronectin-containing extracellular network, depicted as pink molecules in the scheme (Sharma and Rudra, 2018). Finally, adhesion of $CD49d^{high}$ T cells to myoblasts as well as myotubes can also be abrogated by an anti-CD49d monoclonal antibody (Tacke et al., 2009), by consequence inhibiting muscle cell apoptosis. * indicates significant difference with $p < 0.05$.

inflammatory infiltrates, and a higher number of activated $CD49d^{+}HLA\text{-}DR^{+}CD8^{+}$ T lymphocytes in the muscle of patients who had a rapid disease progression (**Figure 1**). Moreover, a higher adhesion of cells obtained from DMD patients to myotubes were observed when compared to healthy controls (Pinto-Mariz et al., 2015).

The importance of $CD49d^{high}$ T lymphocytes in the pathogenesis of the disease has also been observed in the most relevant animal model for this human disease, namely the Golden Retriever muscular dystrophy dog (GRMD). An elevation in the number of circulating $CD4^{+}CD49d^{high}$ T cells at early stages of the disease seems to be highly predictive for the loss of ambulation before 6 months of age (Barthélémy et al., 2014). The same result was not observed with $CD8^{+}CD49d^{high}$ T cells, suggesting that some mechanisms in GRMD may be different from those involved in DMD human patients. In any case, this is an important finding for stratifying these animals for pre-clinical therapeutic studies.

With regard specifically to muscle tissue, an increase in the gene expression of interferon-γ, TGF-β and chemokines, such as CCL14, CCL2, CXCL-12, and CXCL-14 in patients with DMD has been shown (Evans et al., 2009). Considering that extracellular matrix (ECM) elements can interact directly with immune cells also functioning as a substrate for binding soluble factors such as cytokines and chemokines, we could suggest that the combined action between the increase in fibronectin expression, associated with the rise in some chemokines and cytokines could lead to increased recruitment of inflammatory cells to this area. This would result in a perpetual cycle of inflammatory infiltration and deposition of ECM elements, these events being more prominent in the most severe patients.

Considering the studies performed in DMD patients, it is conceivable that the muscle damage initially caused by the absence of dystrophin could result in exposure of antigens on the surface of muscle fibers. Activated T lymphocytes with high expression of CD49d on the surface could migrate to muscle tissue directed by a chemotactic/haptotactic gradient. Moreover, we demonstrated that $CD49d^{hi}$ T cell subsets obtained from DMD patients have a higher *in vitro* migration capacity across endothelial layers and through fibronectin, when compared to $CD49d^{low}$ T subpopulations (Pinto-Mariz et al., 2015). Once in the tissue, $CD8^{+}CD49d^{hi}$ T lymphocytes could recognize antigens on the surface of the fibers causing their destruction.

The higher relative numbers of $CD49d^{hi}$ T cell subsets in the blood of more severe patients, associated with an elevated migratory responsiveness, together with a higher expression of fibronectin and activated $CD8^{+}$ T cells in the muscle, could explain in part the early loss of gait observed in this group of patients.

Overall, effector T cells are important in DMD pathophysiology and evolution with a special role for the $CD49d^{high}$ T cell subpopulations. In this context, $CD49d^{high}$ cells can be used as a prognostic marker of disease progression and CD49d inhibitors can be envisioned as a therapeutic approach to decrease inflammation-mediated tissue damage (see **Figure 1**), with consequent amelioration in the quality of life in DMD patients. As a hope for treating DMD, a clinical trial using siRNA to inhibit CD49d expression in T cells in DMD patients between 10 and 18 years old with loss of deambulation is ongoing and already in phase II[1].

Immune Cross Talk in Skeletal Muscle: The Role of TREG CELLS

As stated above he crosstalk between T cells and macrophages/dendritic cells is essential for immune activation and its maintenance. Although, when shifted to type 1/Th1 immune responses it has a damaging aspect, these same signals are crucial for muscle regeneration, and development. However, if the type 1 response (which includes M1 macrophages and Th1 cells) is not resolved, muscle differentiation does not take place and fibrosis is established (Zhang et al., 2014; Tidball, 2017; Muire et al., 2020).

Fortunately, the immune response has an incredible plasticity and an ongoing response, when healthy, stimulates its own regulation and this is what happens in normal muscle regeneration. Immediately after muscle damage, the type 1 response dominates the scene with the arrival of Th1 lymphocytes and a stimulation of proinflammatory cells. In this phase, proliferation of myoblast progenitors and initial differentiation occurs. This response is crucial for the whole regenerative process and depends on T cells. Genetic deletion of CD8α impairs M1 macrophage infiltration, through the absence of CCL2, leading to a defective muscle regeneration (Zhang et al., 2014). These data indicate the important role of chemokines secreted by $CD8^{+}$ T cells upon monocyte/macrophage attraction to the muscle.

Three to 5 days after the initial injury, there is a shift in the response from a type 1 toward a type 2 (M2 macrophages and Th2 cells)/Treg activity, favoring myoblast fusion and myofiber formation (Riederer et al., 2012).

The signals responsible for this shift are not completely clear, but it is evident that M2 macrophages, secreting TGF-β and IL-10, are crucial for myoblast fusion and maturation (Horsley and Pavlath, 2004). Also, other cells, such as eosinophils, migrate to the lesion and through IL-4/IL-13 secretion modulate fibro-adipogenic precursors (FAP) toward myoblast differentiation supporting fibroblasts (Horsley and Pavlath, 2004; Heredia et al., 2013). IL-4 is also known for its direct role on myoblast fusion through IL-4R present on myoblast and nascent myotubes. Interestingly, this cytokine can be secreted, not only by type 2 immune cells, but also by the myoblasts themselves and the nascent fibers, with a dependence on different NFAT family molecules, similar to the biochemical regulation of T cell differentiation and cytokine secretion (Horsley and Pavlath, 2004).

In parallel to the type 2 response shift, regulatory T cells (Treg) migrate to the lesion and dominate the T cell scenario (Burzyn et al., 2013). Treg cells are $CD4^{+}$ T lymphocytes, characterized by the expression of the transcription factor FoxP3, surface expression of CTLA-4, GITR (the glucocorticoid-induced TNF receptor family related protein) and CD25 – the IL-2 receptor alpha chain).

[1]http://www.anzctr.org.au/Trial/Registration/TrialReview.aspx?ACTRN=12618000970246

They can be generated in the thymus during T cell ontogeny or induced in the periphery of the immune system. These cells have an inhibitory action over most immune cells (including other T lymphocyte subpopulations, DCs, macrophages, B cells) and their effector function depends on antigen recognition, enabling secretion of cytokines, such as TGF-β and IL-10. Moreover, inhibitory activity dependent on cell-to-cell contact, has been reported in the absence of TGF-β or IL-10 (Thornton and Shevach, 1998; Shevach, 2006; Ring et al., 2010; Zhang et al., 2017), reinforcing the notion that various mechanisms underly Treg effector function.

In human inclusion body myositis, it has been shown that Tregs are present in small numbers within the muscle, suggesting that the disease is associated with limited Treg numbers, impacting on the suppression by regulatory T cells over an ongoing inflammatory response (Prevel et al., 2013; Allenbach et al., 2014). In contrast, in juvenile dermatomyositis and DMD, Tregs seem to be increased in the diseased muscle although they are less effective in suppressing an immune response (Vercoulen et al., 2014; Villalta et al., 2014). In line with these findings, a study using a *Toxoplasma gondii* experimental model has shown that muscle from animals infected with the parasite are rich in Treg cells, and that Treg elimination allows M2 differentiation, with consequent improvement in muscle homeostasis, thus suggesting that during this chronic infectious stimulation Tregs gain a different functional phenotype (Jin et al., 2017). This can be the result of a chronic inflammatory environment where Tregs, under chronic IFN-g stimulation may acquire a Th1/Treg phenotype, contributing to inflammation and tissue damage. Together these results show that evaluating only the numbers of Treg's is not sufficient to tell if they are functional or not in chronic muscular diseases. Functional assays are therefore necessary to clarify this issue.

The arrival and maintenance of Tregs in the muscle have been shown to depend on at least two different mechanisms. One of them is dependent on the ATP/P2X axis and was shown in the mdx mouse model of DMD. In this study the authors

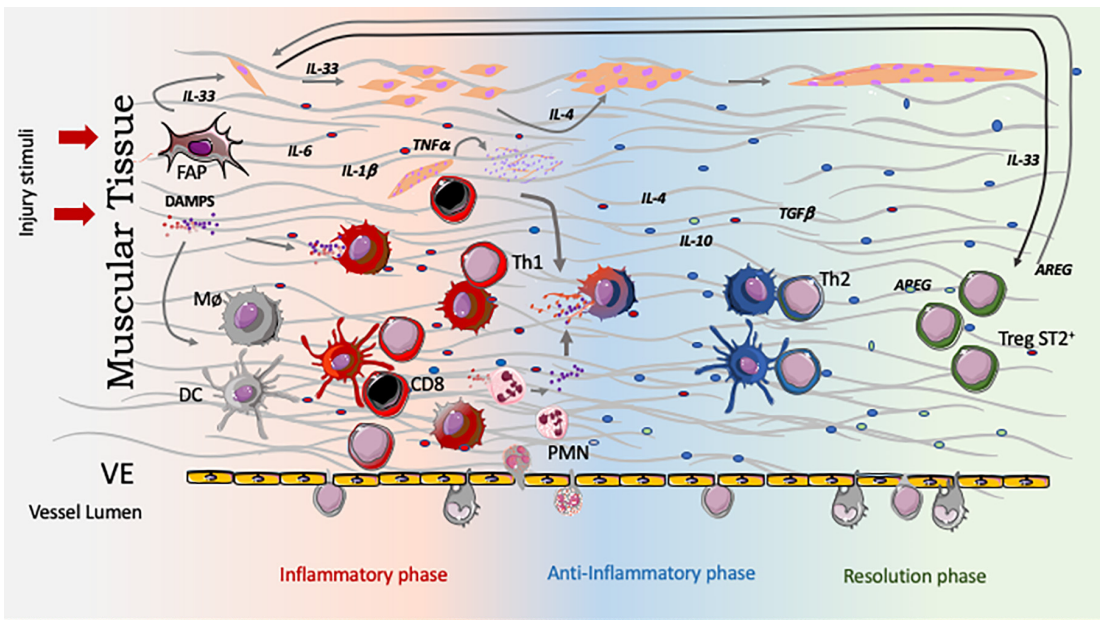

FIGURE 2 | Temporal regulation of the innate and adaptive immune response in muscle injury and healing. Immediately after aggression, muscle cells, as well as fibro-adipogenic precursors (FAPs), produce IL-33. This cytokine induces expansion of the PAX7^{+} satellite cell pool, which themselves also produces IL-33. The necrotic muscle cells release DAMPS, which will stimulate innate immune cells, including resident macrophages and dendritic cells toward a type 1 response represented by the redish colored cells. This initial inflammatory mediator production is guided by cytokines and signals originated from the lesioned muscle. As a result, the whole initial environment is embedded in type 1 cytokines and rapidly incoming cells as neutrophils contribute to the inflammation by several mechanisms. Importantly, after being activated by DAMPs (up to 3 days) both cell types, macrophages and DC, become competent to activate incoming CD4 (Ths) and CD8 T cells keeping, or deviating their function, to a Type 1, building a positive inflammatory loop (this T activation happens in the LN, regardless its possible activation inside the lesioned muscle). Many of the inflammatory cytokines, like TNF-α, IL-1ß, and IL-6 stimulate myoblast progenitor proliferation. After neutrophils come in, attracted by chemokines produced by the vascular endothelium, FAPs and muscle cells themselves, neutrophils are activated, perform their function and evolve to apoptosis. Incoming CD8 cells, besides producing CCL2 which attracts monocytes, will also generate apoptotic bodies by killing damaged muscle cells. Phagocytosis of apoptotic bodies (from neutrophils and muscle) guides macrophages and dendritic cells to a type 2, or type 2 like phenotype(c), contrary to engulfment of necrotic bodies which carry DAMPs. This initial shift is crucial to deviate T cells to a Th2 phenotype. Type 2 responses are linked to IL-4 production which is necessary for myoblast differentiation and fusion. TGF-ß and IL-10 also have roles in the myoblast differentiation and fusion. Interestingly, type one response impairs muscle cell differentiation and maturation. As the healing response progress, together with the shift to type 2 immune response, SP2^{+} Tregs attracted by IL-33, get more numerous and dominates the T cell scenario. Muscle Tregs not only shut down the ongoing immune response but also participate in the healing process, characteristically respond to IL-33 (are ST2^{+} Tregs) producing AREG – a crucial cytokine to expand and maintain the number of satellite cells, in the absence of which regeneration is impaired.

blocked the ATP/P2 × 7 interaction with periodate-oxidized ATP and observed an increased number of Tregs within the injured muscle. Importantly, in the absence of ATP/P2 × 7 signaling, not only the Tregs did arrive in the lesion but muscle damage was reduced, indicating that ATP blocks Treg arrival and in the absence of Treg, muscle damage is more severe (Gazzerro et al., 2015). Much more is known about the IL-33 mediated Treg arrival and maintenance within the muscle. IL-33 is an alarmin produced by FAPs and skeletal muscle stem cell which binds to the interleukin-1 receptor-like 1 protein (also named ST2) present on $CD4^+$ T cells (including Tregs), macrophages and FAPs. In fact, FAP-derived IL-33 is crucial for Treg accumulation within the muscle, and consequently, muscle regeneration (Castiglioni et al., 2015). Muscle T regs, characteristically express ST2 and produce amphiregulin (AREG), a pleiotropic molecule with diverse functions in tissue regeneration and immune suppression (Burzyn et al., 2013; Jin et al., 2018). In the muscle, AREG has been shown to be important for skeletal muscle stem cell expansion in response to IL-33. In addition, muscle Tregs can be modulated by IL-33 producing AREG (Burzyn et al., 2013). Tregs are present in the injured muscle from the very first day, peak between days 3 and 5, and remain in the infiltrate as the majority of T cells until the resolution phase, by day 15). In ageing mice, a deficient production of IL-33 has been shown to be correlated with a diminished migration of Tregs into the muscle whereas treatment with exogenous IL-33 restored the Treg influx and improved muscle regeneration (Kuswanto et al., 2016; Jin et al., 2018).

Regulatory T lymphocytes produce several other cytokines, such as TGF-β and IL-10. Treatment with IL-10 or AREG, both of which are muscle Treg-derived products has been shown to improve muscle repair, as ascertained by an increase in satellite cell and myoblast/myotube markers (Jin et al., 2018). These results strongly suggest that the presence of Tregs contributes to muscle repair.

As mentioned at the beginning of this section, T cells, no matter whether they are CD4 or CD8, T helper or regulatory, need to engage their antigen recognition receptor so that they can signal to other cells. To address this question, Diane Mathis's laboratory showed an increased frequency of one TCR specificity amongst various mouse strains, suggesting that there is an antigen driven accumulation of Tregs in the muscle (Thornton and Shevach, 1998). The same authors made a transgenic mouse carrying this highly frequent TCR (named mTreg24 tg) and showed that Treg accumulation was in fact antigen driven (Cho et al., 2019). More importantly, this model allowed them to study the kinetics of muscle Treg phenotype acquisition with cell transfer experiments. After transfer of the muscle specific T cells to a normal mouse, muscle Treg phenotype ($ST2^+$) was only observed within the muscle, while splenic Tregs, even being specific for muscle antigens, showed the expected splenic Treg signature, with no expression of ST2. Moreover, in the *mdx* model for DMD, mTreg24 provides an accelerated rate of regeneration when compared to polyclonal Treg cells (Cho et al., 2019). It should be noted, however, that such muscle T reg specificity has not yet been studied in humans (Vercoulen et al., 2014; Villalta et al., 2014).

In summary it is very clear that Treg lymphocytes play an important role in the injured/regenerating muscle. Not only do they act as immune suppressors over other immune cells, dampening the inflammatory type 1/Th1 response, but they receive signals from macrophages, FAPs and skeletal muscle stem cells, which in turn, seem to shape their modulatory phenotype inside the muscle. In return, a number of cytokines are secreted by Tregs, favoring muscle stem cell proliferation and progression to myoblast differentiation and fusion in regenerating muscle fibers, if the injury is not chronic. Yet, it should be pointed out that the orchestration of all muscle Treg activities depend on Treg specificity, the only way for T cells to interact long enough with its target, in order to trigger a localized response within the tissue.

CONCLUDING REMARKS

The data discussed above clearly demonstrate the complexity of the cellular and molecular interactions between the skeletal muscle and the immune system, particularly during muscle inflammation. Such a complexity comprises distinct types of interactions, including the production of soluble moieties (cytokines, chemokines), cell-cell interactions mediated by integrin-type ECM receptors, as well as the canonical TCR/MHC-peptide interactions of T lymphocytes with other cells of the immune system or, in certain conditions, with myoblasts themselves. Overall, this complex scenario is schematically depicted in **Figure 2**.

All these interactions take place independently of a given pathogen-derived antigen, being thus a sort of self-within-self recognition at distinct levels of specificity. Accordingly, we think that it should be necessary to take into account all these interaction levels and specificities, when designing cell or gene therapy strategies for correcting genetic or acquired diseases of the skeletal muscle.

AUTHOR CONTRIBUTIONS

AB and WS conceived, participated in writing, and revised the manuscript. CB, FP-M, and IR participated in writing and revised the manuscript. GB-B and VM conceived and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Extracellular ATP Increases Glucose Metabolism in Skeletal Muscle Cells in a P2 Receptor Dependent Manner but Does Not Contribute to Palmitate-Induced Insulin Resistance

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Saturated fatty acids such as palmitate contribute to the development of Type 2 Diabetes by reducing insulin sensitivity, increasing inflammation and potentially contributing to anabolic resistance. We hypothesized that palmitate-induced ATP release from skeletal muscle cells may increase inflammatory cytokine production and contribute to insulin/anabolic resistance in an autocrine/paracrine manner. In C2C12 myotubes differentiated at physiological glucose concentrations (5.5 mM), palmitate treatment (16 h) at concentrations greater than 250 μM increased release of ATP and inflammatory cytokines IL-6 and MIF, significantly blunted insulin and amino acid-induced signaling and reduced mitochondrial function. In contrast to our hypothesis, degradation of extracellular ATP using apyrase, did not alter palmitate-induced insulin resistance nor alter release of cytokines. Moreover, treatment with ATPγS (16 h), a non-hydrolysable ATP analog, in the absence of palmitate, did not diminish insulin sensitivity. Acute treatment with ATPγS produced insulin mimetic roles; increased phosphorylation of PKB (aka AKT), S6K1 and ERK and enhanced GLUT4-mediated glucose uptake in the absence of exogenous insulin. The increases in PKB and S6K1 phosphorylation were completely prevented by pre-incubation with broad spectrum purinergic receptor (P2R) blockers PPADs and suramin but not by P2 × 4 or P2 × 7 blockers 5-BDBD or A-438079, respectively. Moreover, ATPγS increased IL-6 yet decreased MIF release, similar to the cytokine profile produced by exercise. Acute and chronic treatment with ATPγS increased glycolytic rate in a manner that was differentially inhibited by PPADs and suramin, suggesting heterogeneous P2R activation in the control of cellular metabolism. In summary, our data suggest that the palmitate-induced increase in ATP does not contribute to insulin/anabolic resistance in a cell autonomous manner.

Keywords: ATP, skeletal muscle, insulin sensitivity, palmitate, IL-6, MIF, mitochondrial function

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INTRODUCTION

Lipid overflow, particularly of saturated free fatty acids, contributes to the pathophysiology of Type 2 Diabetes (T2D) by reducing insulin sensitivity and enhancing inflammation and mitochondrial dysfunction (Wu and Ballantyne, 2017; Petersen and Shulman, 2018; Sergi et al., 2019). These impairments are predominant in skeletal muscle, resulting in loss of glucose clearance

that accounts for 85–90% of the impairments in glucose homeostasis observed in T2D (Katz et al., 1983; Pendergrass et al., 2007). Lipid-induced muscle insulin resistance (IR) is brought about primarily by impaired insulin signaling due to build-up of lipotoxic species and due to increased levels of circulating pro-inflammatory cytokines. Lipotoxic moieties associated with the development of IR include ceramides (Summers, 2006), long-chain fatty acyl-coenzyme A (Ellis et al., 2000), diacylglycerol (Itani et al., 2002) and acylcarnitines (Aguer et al., 2015), among others [recently reviewed by Bergman and Goodpaster (2020)]. Excess lipid availability also appears to negatively regulate anabolic sensitivity, demonstrated by anabolic resistance in overweight and obese individuals (Guillet et al., 2009; Murton et al., 2015; Beals et al., 2016); in humans following a continuous intralipid infusion (Stephens et al., 2015), in diet-induced obese rodents (Anderson et al., 2008; Masgrau et al., 2012) and in high-fat fed men in a model of disuse-induced IR (Wall et al., 2020).

A proportion of skeletal muscle inflammation occurs due to immune cell infiltration of muscle adipose depots (Khan et al., 2015). Independently from this, treatment with saturated fatty acids, particularly palmitate, as the major dietary fatty acid in blood, enhances the release of cytokines such as interleukin 6 (IL-6) and activates the IKK/NF-κB signaling cascade (Jove et al., 2005), which is associated with the development of skeletal muscle IR. IL-6 has been extensively characterized for its paradoxical roles in inflammation. Acute and transient exercise-induced increases in IL-6 contribute to the regulation of glucose homeostasis, for example, by stimulating glucose production (Fischer, 2006; Pedersen and Febbraio, 2008; Wolsk et al., 2010; Eckardt et al., 2014), glucagon secretion (Barnes et al., 2014) and altering adipose tissue function (Brandt et al., 2012). In contrast, chronically elevated circulating IL-6 features in pro-inflammatory conditions and is associated with muscle wasting and IR (Kern et al., 2001; Pradhan et al., 2001). Other cytokines and myokines, such as macrophage migration inhibitory factor (MIF), are elevated in inflammatory conditions such as obesity (Dandona et al., 2004) and T2D (Yabunaka et al., 2000) and have modulatory roles in glucose homeostasis (Atsumi et al., 2007; Miyatake et al., 2014). It remains important to establish the autocrine and paracrine roles of these myokines in skeletal muscle IR and inflammation.

Palmitate can also enhance muscle ATP release in a pannexin hemi-channel-mediated manner (Pillon et al., 2014). Palmitate-induced extracellular ATP (eATP) acts as a chemoattractant for immune cells, suggesting that the nucleotide plays an important role in the immune cell infiltration seen in conditions characterized by chronic low-grade inflammation, such as obesity and T2D (Wu and Ballantyne, 2017). In other tissues, eATP (and degradation products) has been implicated in inflammation and IR, as seen in adipose tissue (Tozzi and Novak, 2017; Wu and Ballantyne, 2017); in blockade of insulin receptor signaling in hepatocytes by adenosine diphosphate (ADP) (Chatterjee and Sparks, 2012) and by impaired glucose clearance and hepatic insulin sensitivity in mice lacking the ectonucleotidase CD39 (Enjyoji et al., 2008).

ATP is released from skeletal muscle in response to electrical stimulation (Buvinic et al., 2009; Bustamante et al., 2014) and by contraction/exercise (Steensberg et al., 2000; Osorio-Fuentealba et al., 2013). eATP acts in an autocrine/paracrine manner by signaling via metabotropic P2Y and ionotropic P2X purinergic receptors (P2Rs) leading to increased intracellular calcium concentration ($[Ca^{2+}]_i$) (Cseri et al., 2002; Abdul-Ghani et al., 2008; Ito et al., 2018) and transactivation and transinhibition of pathways such as MAPK/ERK and protein kinase C signaling cascades (Ito et al., 2018). eATP also acts as an important vasodilator (Mortensen et al., 2009) and likely contributes to muscle hypertrophy following exercise (Ito et al., 2018). Given the well-characterized pro-inflammatory roles of eATP in other tissues and enhanced ATP release following treatment with saturated fatty acids, we hypothesized that palmitate-induced increases in eATP may contribute to the development of insulin/anabolic resistance and inflammation in an autocrine/paracrine manner in skeletal muscle, in the absence of immune cells.

MATERIALS AND METHODS

Chemicals

Novo Nordisk Actrapid recombinant human insulin was used for cell treatments (Henry Schein, United Kingdom). ATPγS, PPADs, suramin, 5-BDBD, A-438079 were purchased from Tocris (Bristol, United Kingdom). Fluo-4 Direct and 50 x MEM amino acid solution were from Thermo Fisher Scientific (Loughborough, United Kingdom). ATP (adenosine 5'-triphosphate magnesium salt), apyrase, fatty acid-free BSA, palmitic acid and indinavir were purchased from Sigma Aldrich (Poole, United Kingdom).

Cell Culture

The C2C12 (ATCC CRL-1772, *Mus musculus*) murine myoblast cell line was kindly gifted by colleagues at the University of Dundee. Cell were maintained in growth medium below 70% confluence, incubated at 37°C in humidified 5% CO_2 incubators. Cells were differentiated at physiologically relevant glucose concentrations (5.5 mM). For differentiation, cells were plated in plating medium and incubated for 48 h (reaching 100% confluence) before medium was changed to differentiation medium. Differentiation medium was changed every day for 6 consecutive days to replenish glucose. Experiments were conducted after 7 days of differentiation. Refer to **Supplementary Methods** for media composition and seeding densities.

Immunoblotting

Experiments in 60 mm dishes were terminated by media collection (for ATP and cytokine analysis) and cell lysis for protein extraction. Protein concentrations were determined by the Bradford method and lysates subjected to SDS-PAGE and electrotransferred to nitrocellulose membranes. Total and phosphorylated proteins and loading controls were immunoblotted and semi-quantified by infrared fluorescence using the Licor Odyssey scanner. See **Supplementary Methods** for lysis buffer and antibody details.

Cytotoxicity Assessment

Cell viability was assessed in C2C12 myotubes using extracellular lactate dehydrogenase (LDH) activity measured using a commercially available kit and according to manufacturer's instructions (LDH assay kit, Abcam, United Kingdom). Briefly, C2C12 myoblasts were differentiated as above for 6 days and treated with palmitate (0–750 μM) or BSA controls for 16 h before LDH activity was measured. Medium collected after overnight treatment was centrifuged (600 × *g* for 10 min) and supernatants incubated with WST and lactate dehydrogenase. LDH oxidizes lactate (producing NADH) which reacts with WST to generate a yellow color which was detected by absorbance (OD_{450nm}) using Pherastar FS plate reader.

ATP Quantification

The extracellular concentration of ATP was measured using a commercially available assay kit and used according to manufacturer's instructions (ATPLite, PerkinElmer) and as previously described (Vlachaki Walker et al., 2017). See **Supplementary Methods** for details.

Assessment of Cellular Metabolism

Mitochondrial and glycolysis stress tests were performed according to manufacturer's instructions with minor modifications (Agilent, United Kingdom). Briefly, for mitochondrial stress tests, cells were treated for 16 h with palmitate or BSA controls and treated acutely with with oligomycin (2 μM), FCCP (1 μM) and rotenone/antimycin A (1:1 ratio, final concentration 1 μM). For glycolysis stress tests, cells were treated with ATPγS (up to 16 h) and treated acutely with glucose (10 mM); oligomycin (2 μM) and 2-deoxyglucose (2-DG, 50 mM). Refer to **Supplementary Methods** for details.

Cytokine Quantification

The levels of extracellular cytokines present in conditioned media from treated cells were quantified using enzyme linked immunosorbent assays (ELISAs). MIF and IL-6 were measured using mouse Duo-set ELISA kits (Bio-Techne, Abingdon, United Kingdom), performed according to manufacturer's instructions.

Measurement of Intracellular Calcium

Changes in intracellular calcium were assessed using the fluorescent calcium indicator Fluo-4 Direct as previously described (Ito et al., 2018), with minor modifications for the C2C12 cells. Briefly, C2C12 myotubes were differentiated in clear, flat bottomed 96 well plates. After 7 days, cells were incubated with Fluo-4 Direct-containing phenol red free medium for 60 min and relative changes in fluorescence measured using a Pherastar FS plate reader. See **Supplementary Methods** for details.

Measurement of Glucose Uptake

Briefly, C2C12 myotubes were differentiated in 96-well plates for 7 days (as above). For palmitate experiments, cells were treated for 16 h with palmitate (500 μM) or BSA control on day 6. On day 7, cells were serum starved for 2 h and glucose starved for 1–2 h before incubation with treatments and subsequent incubation with 2-DG (100 μM, 15 min). 2-DG uptake into C2C12 myotubes was assessed using the Glucose Uptake-Glo assay (Promega, Southampton, United Kingdom). See **Supplementary Methods** for details.

Statistical Analyses

In Western blotting experiments, a One−sample *t*−test was used to determine significant changes in phosphorylation or total protein expression, relative to control (normalized to 1). For multiple group comparisons, a one−way or two-way analysis of variance with *post hoc* Bonferroni were used. Statistical tests were performed using GRAPHPAD PRISM software (Prism 7; GraphPad Software, La Jolla, CA, United States). Results are expressed as mean ± standard error. P values < 0.05 were taken to indicate statistical significance.

RESULTS

Palmitate Treatment Diminishes Insulin Sensitivity and Increases ATP Release

Differentiation of myoblasts into myotubes at physiologically relevant glucose concentrations yielded myotubes that were sensitive to insulin (10–100 nmol/L), as measured by significant increases in PKB and S6K1 phosphorylation (**Figures 1A,B**). In addition, treatment with mixed essential amino acids (AA) enhanced S6K1 phosphorylation (**Figure 1C**). In the absence of palmitate, insulin and AAs significantly increased the levels of phosphorylated PKB and S6K1, however, in the presence of 500 μM palmitate, insulin/AA-induced PKB and S6K1 phosphorylation was significantly attenuated (**Figures 1Di–iii**). We observed no change in extracellular ATP (eATP) levels with 250 μM palmitate, but at 500 μM palmitate, eATP levels were elevated approximately two-fold (**Figure 1E**). Treatment with 500 μM palmitate did not alter cell viability (**Figure 1F**). As expected, palmitate treatment led to significant impairments in mitochondrial function with cells displaying reduced basal oxygen consumption rates (OCR; **Figures 1Gi,ii**), reduced oligomycin-sensitive OCR (a measure of ATP production; **Figures 1Gi,iii**), diminished spare respiratory capacity (**Figures 1Gi,iv**), increased proton leak (**Figures 1G,Gv**) and reduced coupling efficiency (**Figure 1Gvi**).

Degradation of eATP Does Not Alter Palmitate-Induced Insulin Resistance and Cytokine Release

In separate studies, we confirmed that palmitate, at pro-inflammatory concentrations, enhanced eATP and that apyrase degraded palmitate-induced eATP by 90% (**Figure 2A**). Palmitate treatment significantly blunted insulin and AA-mediated phosphorylation of PKB and S6K1, however, apyrase treatment did not alter blunting of insulin/AA-mediated signaling (**Figures 2Bi–iv**). Palmitate significantly increased ERK1/2 phosphorylation and this was not altered by apyrase,

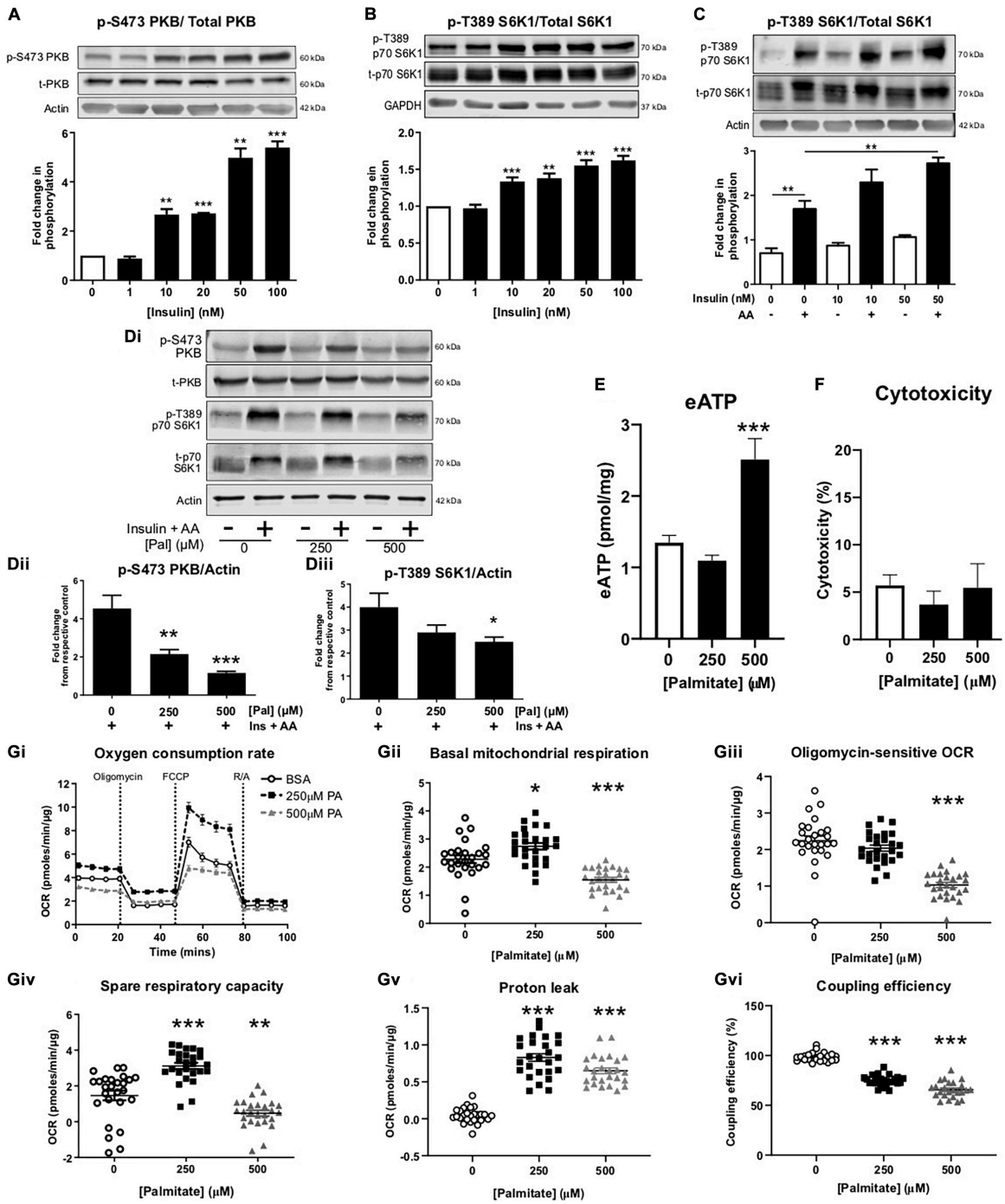

FIGURE 1 | Palmitate induces insulin resistance, enhances extracellular ATP and impairs mitochondrial function in C2C12 myotubes. Representative immunoblots for PKB (pS473) normalized to PKB **(A)** and p70-S6K1 (pT389) normalized to p70-S6K1 **(B)** with densitometric analysis below for cells treated with insulin (0–100 nM) for 60 min (n = 4–7). Actin and GAPDH used as loading controls. **(C)** Representative immunoblot for p70-S6K1 (pT389) normalized to p70-S6K1 for cells treated for 60 min with amino acids (AA, 3.34 mM) or combined with insulin (0–50 nM) (n = 3). **(Di)** Representative immunoblots for PKB (pS473) and p70-S6K1 (pT389) normalized to actin with densitometric analysis in **(Dii,iii)**, respectively, represented as fold change from respective control, for cells treated with palmitate (0–500 μM; 16 h) and insulin (20 nM) and amino acids (AA, 3.34 mM) for 60 min (n = 5–6). **(E)** Extracellular ATP measured by luminescence after palmitate treatment (0–500 μM; 16 h; n = 7). **(F)** Cytotoxicity assessed by extracellular lactate dehydrogenase in palmitate (0–500 μM; 16 h) treated myotubes (n = 3). **(Gi)** Representative extracellular flux assay demonstrating oxygen consumption rate (OCR; pmoles/min/μg) at baseline and after acute injection of oligomycin (2 μM), FCCP (1 μM) and rotenone/antimycin A (R/A; 1:1 ratio; 1 μM) at the indicated stages (n = 26–28). **(Gii)** Mean basal OCR before oligomycin. **(Giii)** Oligomycin-sensitive OCR measured as effect of oligomycin. **(Giv)** Spare respiratory capacity, measured as FCCP effect. **(Gv)** Proton leak measured as R/A effect. **(Gvi)** Coupling efficiency measured as the ratio between oligomycin-sensitive OCR and mitochondrial basal OCR expressed as percentage. *P < 0.05, **P < 0.01, ***P < 0.001 against untreated control.

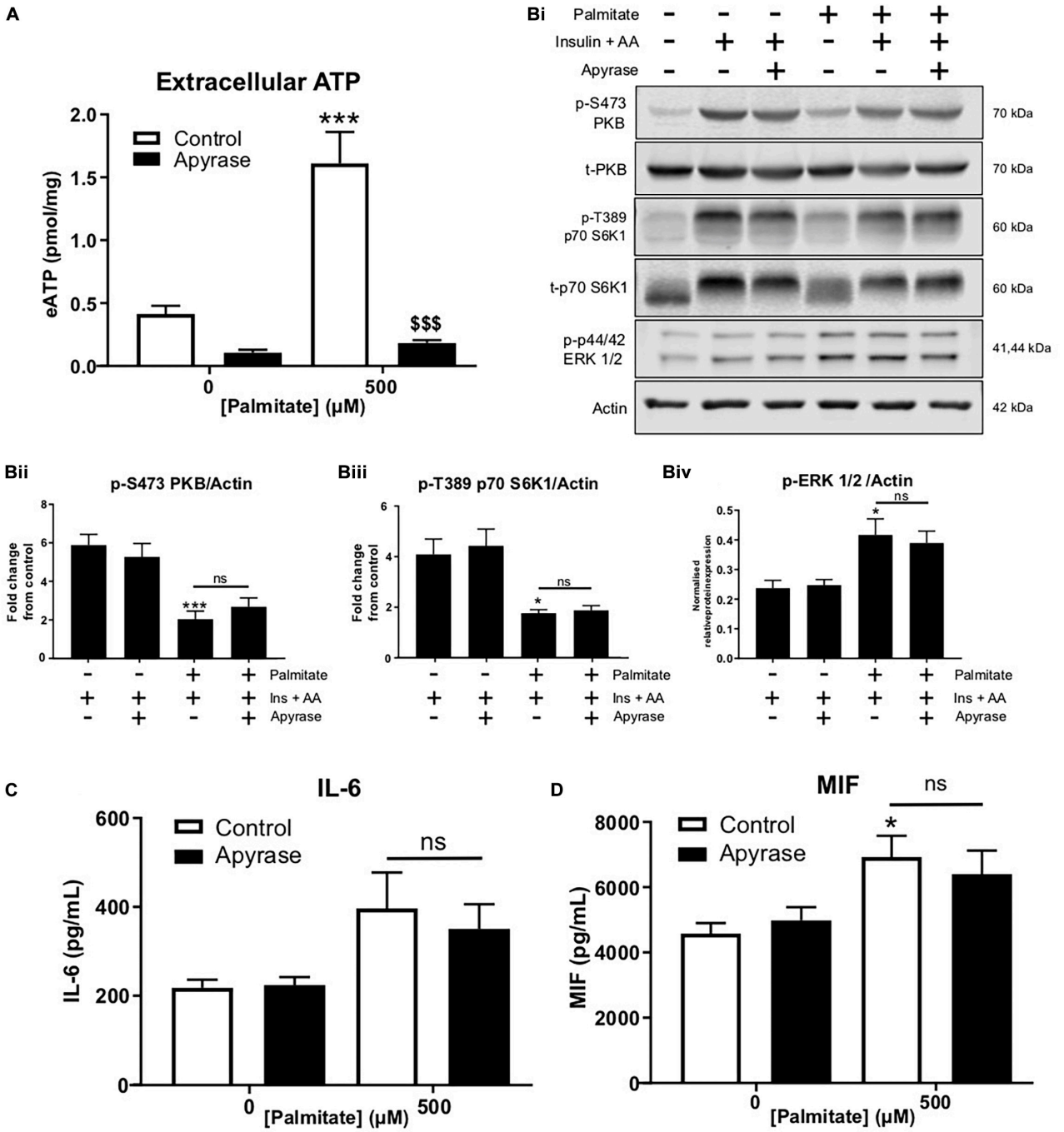

FIGURE 2 | Palmitate-induced insulin and anabolic resistance and cytokine release is not altered by hydrolysis of extracellular ATP with apyrase in C2C12 myotubes. **(A)** Palmitate-induced extracellular ATP (500 μM; 16 h) hydrolysed by treatment with apyrase (0.5 units/mL; 16 h) in cells treated for 15 h with PA + apyrase and 1 h with insulin (20 nM) and amino acids (3.34 mM) (n = 6). **(Bi)** Representative immunoblots for PKB (pS473), total PKB, p70-S6K1 (pT389), p70-S6K1, ERK 1/2 (pThr202/Tyr204) and actin (n = 6) for cells treated as in A. Densitometric analysis for PKB (pS473) normalized to PKB **(Bii)** p70-S6K1 (pT389) normalized to p70-S6K1 **(Biii)** and ERK 1/2 (pThr202/Tyr204) normalized to actin **(Biv)**. **(C,D)** Hydrolysis of extracellular ATP with apyrase (0.5 units/mL; 16 h) did not alter the palmitate (500 μM; 16 h)-induced increase in release of cytokines IL-6 (C; n = 6) or MIF (D; n = 5). $^{*}P < 0.05$, $^{***}P < 0.001$ for palmitate against control and ns = not significant for the effect of apyrase on palmitate response. $^{\$\$\$}P < 0.001$ against palmitate treated group.

suggesting that additional mechanisms contribute to the palmitate-induced phosphorylation of ERK1/2 (**Figures 2Bi**, iv). We noted a modest increase in IL-6 and a significant increase in macrophage migration inhibitory factor (MIF) induced by palmitate but this was not significantly modified by apyrase (**Figures 2C,D**). Taken together these data indicate that eATP

does not contribute to palmitate-induced insulin resistance nor does it significantly contribute to palmitate-induced cytokine release from skeletal muscle.

ATPγS Treatment in the Absence of Palmitate Does Not Alter Insulin Sensitivity but Does Alter Cytokine Secretion

To confirm ATP-induced signaling in C2C12 myotubes, intracellular calcium responses were monitored following ATP treatment. ATP caused a concentration-dependent increase in intracellular calcium, indicating P2R-mediated signaling (**Figures 3A,B**). To determine whether enhanced P2R-mediated signaling could alter insulin sensitivity, in the absence of palmitate, myotubes were incubated with a non-maximal concentration of ATPγS (based on changes to intracellular calcium levels), a non-hydrolysable ATP analog, for 16 h prior to acute insulin treatment. As expected, acute insulin treatment increased PKB and S6K1 phosphorylation but this was not modified by ATPγS pre-treatment (**Figures 3C,D**). Interestingly, when measuring release of IL-6 and MIF from C2C12 cells treated with ATPγS, we observed a significant concentration-dependent increase in IL-6 release (**Figure 3E**). In contrast to the palmitate response, however, we observed a significant reduction in MIF release following ATPγS treatment (**Figure 3F**).

Acute ATPγS Treatment Increases PKB and S6K1 Phosphorylation in a P2R-Dependent Manner and Enhances GLUT4-Mediated Glucose Uptake in Healthy but Not Palmitate-Challenged Cells

To examine ATPγS signaling in more detail, C2C12 myotubes were treated acutely with ATPγS and PKB and S6K1

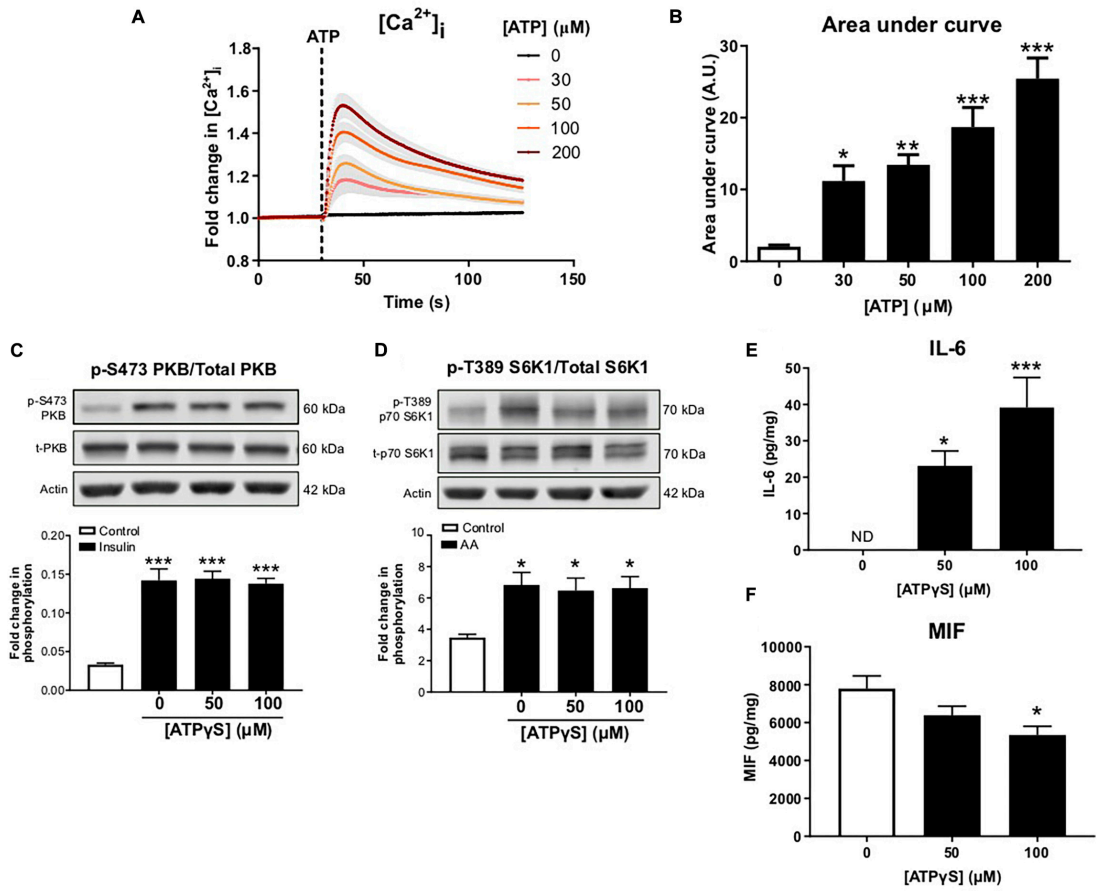

FIGURE 3 | Treatment with exogenous ATP increases intracellular calcium and alters cytokine release without impairing anabolic sensitivity in C2C12 myotubes. **(A)** Cells loaded with Fluo 4 direct for 60 min. Concentration-dependent increase in intracellular calcium assessed by change in fluorescence (RFU) with ATP (0–200 μM; 30 s) with area under the curve in **(B)** (n = 6). **(C)** Representative immunoblot and densitometric analysis for PKB (pS473) normalized to PKB following 16 h treatment with 50–100 μM ATPγS and 1 h treatment with insulin (20 nM) or control (n = 6). **(D)** Representative immunoblot and densitometric analysis for p70-S6K1 (pT389) normalized to p70-S6K1 following 16 h ATPγS treatment and 1 h treatment with mixed essential amino acids (AA, 3.34 mM) or control (n = 6). **(E,F)** Exogenous ATPγS (50–100 μM; 16 h) enhanced IL-6 release (E; n = 6) and suppressed MIF release (F; n = 6). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ against vehicle control.

phosphorylation was examined. Correlating with recent studies (Osorio-Fuentealba et al., 2013; Ito et al., 2018), we observed significant increases in phosphorylation of both PKB and S6K1 following ATPγS treatment (**Figures 4A,B**). To determine which P2Rs may be responsible, myotubes were pre-incubated with broad-spectrum P2R blockers PPADs and suramin, in addition to P2 × 4 and P2 × 7 antagonists, 5-BDBD and A-438079. Interestingly, PPADs significantly blocked ATPγS-induced increases in PKB, S6K1 and ERK1/2 phosphorylation while suramin significantly attenuated the latter two and modestly but not significantly attenuated PKB phosphorylation. Moreover, basal phosphorylation was also reduced, indicating tonic receptor activation (**Figures 4Ci–iv**). Acute treatment with ATPγS enhanced glucose uptake in a concentration dependent manner, independently of insulin (**Figure 4D**). In addition, pre-treatment with GLUT4 inhibitor

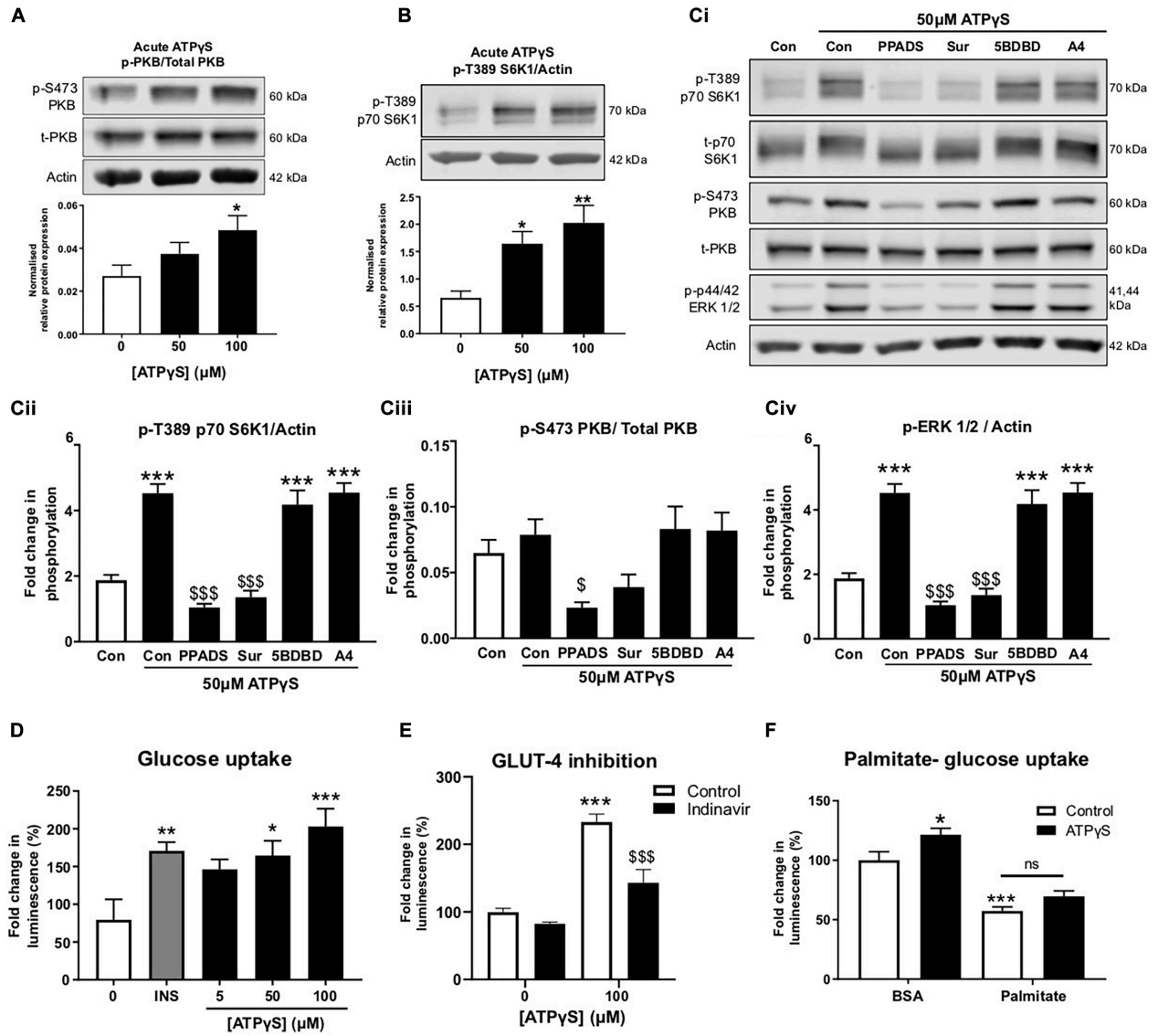

FIGURE 4 | ATPγS has insulin-mimetic roles in C2C12 myotubes activating PI3K/PKB and mTOR pathway signaling proteins in a P2-receptor-dependent manner and enhancing GLUT4-mediated glucose uptake. **(A,B)** Representative immunoblots for PKB (pS473) normalized to PKB (A; $n = 5$) and p70-S6K1 (pT389) normalized to p70-S6K1 (B; $n = 6$) with densitometric analysis below for cells treated with ATPγS (50–100 μM; 15 min). **(Ci)** Representative immunoblots for PKB (pS473), PKB, p70-S6K1 (pT389), p70-S6K1, ERK 1/2 (pT202/TY204) and actin for cells treated with ATPγS (50 μM; 15 min) with and without broad spectrum P2 receptor antagonists PPADs (100 μM) and suramin (100 μM), P2X4R antagonist 5-BDBD (5 μM) and P2X7R antagonist A438079 (100 μM) (45 min prior to spiked ATPγS; $n = 6$). Densitometric analysis for p70-S6K1 (pT389) normalized to p70-S6K1 in **(Cii)**; PKB (pS473) normalized to PKB in **(Ciii)** and ERK 1/2 (pT202/TY204) normalized to actin in **(Civ)**. **(D)** Glucose uptake (2-DG; 100 μM; 15 min) assessed by luminescence following treatment with ATPγS (0–100 μM; 15 min pre-treatment) or insulin (200 nM; 15 min pre-treatment) ($n = 9$). **(E)** Glucose uptake (2-DG; 100 μM; 15 min) following treatment with ATPγS (100 μM; 15 min) in the presence or absence of GLUT4 inhibitor indinavir (50 μM) ($n = 5$). **(F)** Glucose uptake (2-DG; 100 μM; 15 min) following treatment with palmitate (500 μM; 16 h) and ATPγS (100 μM; 15 min) ($n = 17$). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ against untreated control, $\$\$\$P < 0.001$ against ATPγS control.

indinavir attenuated ATPγS-mediated glucose uptake by 40% (**Figure 4E**). To assess whether ATPγS-mediated glucose uptake was sustained in insulin resistant myotubes, we assessed glucose uptake following palmitate treatment and demonstrated that palmitate significantly attenuated basal and ATPγS-stimulated glucose uptake (**Figure 4F**).

ATPγS Increases Glycolytic Metabolism by a P2R-Dependent Mechanism

To examine whether the enhancement in glucose uptake led to altered intracellular glucose metabolism, we performed a glycolysis stress test in the presence and absence of ATPγS and P2R antagonists. Acute injection of ATPγS induced a modest but statistically significant increase in the extracellular acidification rate (ECAR), in the absence of glucose (**Figures 5Ai,ii**). This response was abolished by PPADs but not by suramin, indicating this acute response is unlikely to be mediated by purely the addition of ATPγS. In the presence of suramin, ATPγS generated a large increase in ECAR (**Figures 5Ai,ii**). This was not accompanied by increased oxygen consumption rate (OCR; data not shown) suggesting this is not mediated by CO_2 production from the mitochondria. On addition of glucose, ATPγS treatment augmented the ECAR response, suggesting enhanced glycolysis. Again, this was blocked by PPADs, but not by suramin (**Figures 5Ai,iii**). We next examined the effect of chronic (16 h) ATPγS treatment on glycolytic function. Correspondingly, ATPγS significantly enhanced glycolysis. In this paradigm, pre-incubation with suramin prevented the enhanced ECAR response to glucose injection by ATPγS, producing ECAR levels similar to control (**Figures 5Bi,ii**). Non-glycolytic acidification (before injection of glucose) was not altered by ATPγS pre-treatment (**Figure 5Biii**).

DISCUSSION

Skeletal muscle insulin resistance contributes substantially to the pathophysiology of T2D. Defining the pathways of insulin resistance and uncovering potential mechanisms to improve insulin sensitivity are key therapeutic areas requiring further investigation. It has previously been reported that palmitate induces release of ATP in skeletal muscle, which can recruit immune cells (Pillon et al., 2014). In liver, saturated fatty acids also increase hemi-channel expression to increase ATP release which can stimulate apoptosis (Xiao et al., 2012). Moreover, the action of ATP at P2Rs is a potent modulator of pro-inflammatory cytokine production and release in a number of non-immune cells including keratinocytes (Kawano et al., 2015), endothelial cells (Seiffert et al., 2006) and astrocytes

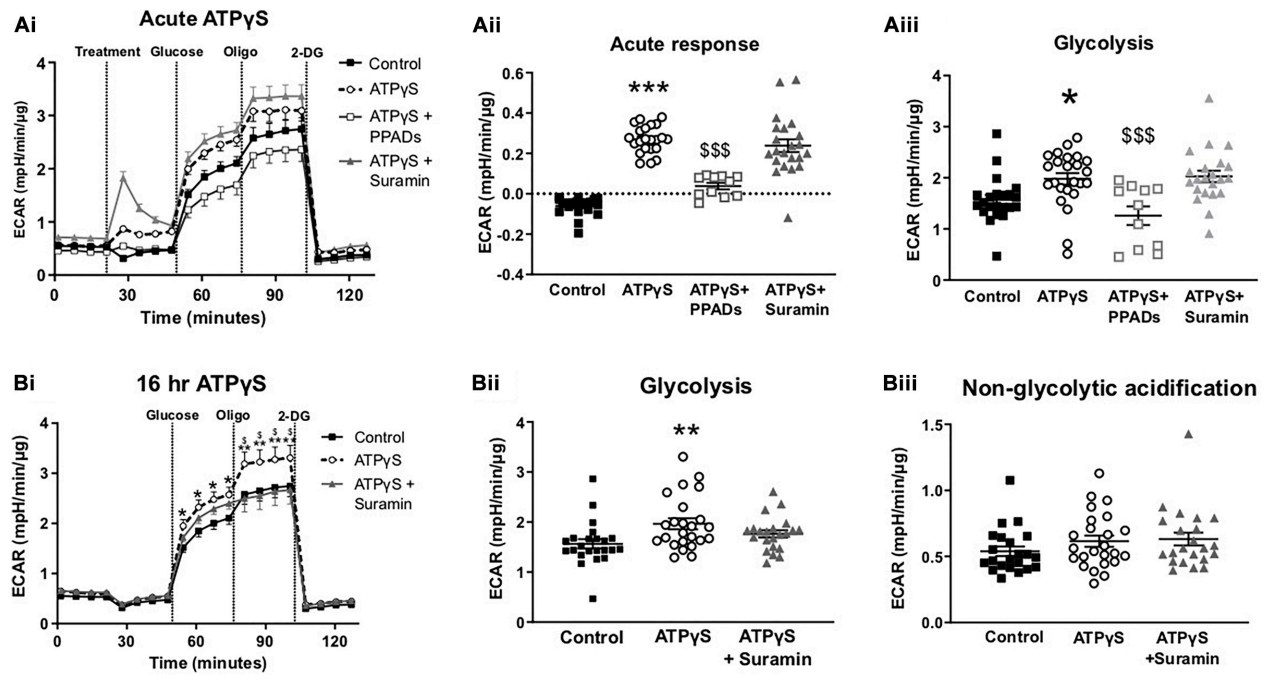

FIGURE 5 | Treatment with ATPγS enhances extracellular acidification rate in C2C12 myotubes in a P2-receptor-dependent and insulin-independent manner. **(Ai)** Representative extracellular flux assay demonstrating changes to glycolytic rate as estimated by extracellular acidification rate (ECAR) in response treatment with ATPγS (100 pM; treatment) with or without suramin (100 pM), PPADs (100 pM) or vehicle; followed by acute injection of glucose (5.5 mM), oligomycin (2 pM) and 2DG (50 mM) at the indicated points (n = 22–24). **(Aii)** Acute response, ECAR prior to glucose **(Aiii)** Glycolysis measured as the change in ECAR following glucose injection. **(Bi)** Representative extracellular flux assay for cells treated with ATPγS (100 pM; 16 h) with or without suramin (100 pM) or vehicle and acute injection of glucose (5.5 mM), oligomycin (2 pM) and 2-DG (50 mM) at the indicated points (n = 22–24). **(Bii)** Glycolysis measured as the change in ECAR following glucose injection. **(Biii)** Non-glycolytic acidification demonstrating mean ECAR prior to glucose injection. Data represented as mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ against control, $\$P < 0.05$, $\$\$\$P < 0.001$ against ATPγS.

(Liu et al., 2000). Therefore, given the role of ATP in mediating pro-inflammatory signaling, we postulated that ATP release from skeletal muscle, in an autocrine/paracrine manner and independently of immune cell recruitment, may contribute to production of pro-inflammatory cytokines and reduced insulin sensitivity in palmitate challenged myotubes. Here we utilized the C2C12 myoblast cell line, differentiated into myotubes, to observed ATP release and exogenous ATP signaling in a cell autonomous manner. We demonstrate that palmitate induces ATP release from skeletal muscle myotubes independently of cell death, assessed by measurement of extracellular lactate dehydrogenase, agreeing with previous reports demonstrating palmitate-induced ATP release in a regulated manner (Pillon et al., 2014). In our study, palmitate-induced ATP release was accompanied by reduced insulin/AA sensitivity, diminished mitochondrial function and increased release of cytokines IL-6 and MIF. Contrary to our hypothesis, degradation of eATP using apyrase did not modify palmitate-induced insulin resistance nor cytokine release. In contrast, enhanced eATP signaling (in the absence of palmitate) produced insulin mimetic actions; increasing PKB, S6K1 and ERK1/2 phosphorylation. Recently, Ito and colleagues observed eATP-mediated increases in S6K1 and protein synthesis in C2C12 myotubes, mediated by the P2Y2 receptor (Ito et al., 2018). Our findings are in line with these observations and suggest that eATP has anabolic actions in muscle.

We also observed increased GLUT4-mediated glucose uptake into C2C12 myotubes following ATPγS treatment, in line with previous reports (Osorio-Fuentealba et al., 2013). Exercise significantly increases ATP demand/turnover in muscle (Gaitanos et al., 1993) and palmitate-induced mitochondrial dysfunction leads to reduced ATP production (Nisr and Affourtit, 2016) and, interestingly, ATP release occurs in both situations (Osorio-Fuentealba et al., 2013; Pillon et al., 2014). Contraction and exercise-induced ATP release contributes to glucose uptake and cytokine release in skeletal muscle (Osorio-Fuentealba et al., 2013; Bustamante et al., 2014). If sensitivity to ATP-induced glucose uptake is sustained in insulin resistant states, this may contribute to exercised-induced anabolism and provide a novel therapeutic strategy to maintain glucose homeostasis in T2D. We tested the acute response to ATPγS following palmitate treatment, mimicking contraction-induced ATP release in insulin resistant conditions, and demonstrated attenuated ATP-simulated uptake in palmitate-challenged cells, suggesting a level of purinergic resistance. Resistance to ATP-mediated glucose uptake has been demonstrated at lower ATP concentrations in muscle fibers from high-fat fed mice, but not at higher ATP concentrations, where response is sustained {Osorio-Fuentealba et al., 2013 #5679}. Additional experimentation is required to investigate purinergic sensitivity in metabolically impaired skeletal muscle. However, others have demonstrated diminished P2R responses to ATP in insulin resistant conditions, such as dampened P2Y2R-mediated vasodilation in T2D (Thaning et al., 2010), indicating that purinergic resistance could also occur at the level of glucose uptake and metabolism.

Our novel observations that ATPγS enhances skeletal muscle cell glycolytic function suggests an important receptor-mediated process to increase ATP generation as a positive feedback mechanism. Similar increases in cellular metabolism have been reported previously in endothelial cells, where exogenous ATP increased ECAR and upregulated key glycolytic enzymes including hexokinase, glucose transporter 1 expression and phosphofructokinase B3 (Lapel et al., 2017). This effect of eATP on metabolism is likely tissue specific as eATP has been reported to decrease cellular oxygen consumption in kidney cells, in a manner sensitive to suramin (Silva and Garvin, 2009). It is also worth noting that PPADs and suramin differentially blocked the effect of ATPγS on metabolism in this study, indicating that both P2X and P2YRs may contribute to glucose uptake and/or metabolism. Interestingly, blockade of G-protein signaling only partially attenuated ATP-induced glucose uptake (Osorio-Fuentealba et al., 2013), suggesting a component of glucose transport could be P2XR mediated. However, it is important to note that purinergic receptor and ATPase expression can change during differentiation (Martinello et al., 2011) and varies across species (Abdelmoez et al., 2020), therefore determining the receptor(s) in human skeletal muscle going forward will be important.

In our study, ATPγS also increased IL-6 and decreased MIF release from myotubes. In general, transient and short-term muscle-derived IL-6, such as seen during exercise, promotes hypertrophy, myogenesis and regeneration (Fischer, 2006). IL-6 can also enhance skeletal muscle glucose uptake and fat oxidation via AMP-activated protein kinase dependent and independent pathways (Carey et al., 2006; Wolsk et al., 2010; Ikeda et al., 2016). Conversely, long-term elevation of IL-6, which is evident in inflammatory conditions, is associated with muscle wasting and IR (Kern et al., 2001; Pradhan et al., 2001; Rui et al., 2002). Therefore it is plausible that persistent fatty acid-induced ATP release drives chronic IL-6 signaling to contribute to IR in an autocrine/paracrine fashion, in addition to the recruitment of immune cells (Pillon et al., 2014). Therefore the time course and persistence of the ATP release likely contributes to the differential response to acute (exercise-induced) versus chronic (palmitate-induced) ATP release. For example, a sustained elevation in eATP may be required to recruit and polarize immune cells toward a pro-inflammatory phenotype (Desai and Leitinger, 2014; Pillon et al., 2014). Furthermore, the physiological (increased ATP demand during contraction) and pathological context (impaired ATP production during excess lipid) may alter the outcome of eATP signaling, which requires further investigation.

Interestingly, ATPγS, in the absence of palmitate, decreased MIF release. MIF is a pro-inflammatory cytokine that is packaged into readily releasable pools (Bacher et al., 1997). The circulating levels of MIF are elevated in T2D (Yabunaka et al., 2000), Type 1 Diabetes (Bojunga et al., 2003; Ismail et al., 2016) and a number of other autoimmune diseases (Ayoub et al., 2008; Vincent et al., 2019). In addition, deletion of MIF in pancreatic beta cells protects them against palmitate-induced apoptosis (Saksida et al., 2012). In adipose tissue, MIF can inhibit insulin-stimulated glucose uptake (Atsumi et al., 2007) and in skeletal muscle, MIF can reduce insulin and AICAR-mediated glucose transport (Miyatake et al., 2014). Therefore, the significant decrease in MIF

release following ATPγS treatment suggests a possible beneficial effect of ATPγS.

Although not yet explored, it is also plausible that the benefits of neuromuscular electrical stimulation (NMES) in preventing disuse-induced atrophy during immobilization (Dirks et al., 2014) are partly attributed to the autocrine anabolic roles of eATP. This contraction-induced ATP release to the muscle interstitium (Mortensen et al., 2009) is mirrored by a transient increase in plasma ATP which increases and returns to basal levels within 30 min of recovery (Zarêbska et al., 2018). Utilizing ATP as a signal in this manner has a number of advantages specifically in muscle. For example, muscle contraction causes plasma membrane damage (McNeil and Khakee, 1992), leading to release of intracellular contents (including ATP); differentiated muscle cells express high levels of ATPases (Martinello et al., 2011) that rapidly degrade eATP allowing swift termination of the signal; and eATP can contribute to enhanced blood flow and oxygenation into muscle (Rosenmeier et al., 2008; Mortensen et al., 2009), which is essential during exercise. Whether this can be exploited as a therapy remains to be determined. Distinguishing physiological versus pharmacological actions of ATP will be important going forward, particularly during different states such as exercise or nutrient excess. However, in humans, oral ATP supplementation has been reported to increase muscle blood flow (Jäger et al., 2014) and size following resistance training (Wilson et al., 2013). It can also promote hypotension after exercise in hypertensive women (de Freitas et al., 2018). The reasons for the benefits of oral ATP remain unknown given that the bioavailability of oral ATP is very low (Arts et al., 2012) and that direct infusion of ATP into the femoral vein failed to increase interstitial ATP concentrations (Mortensen et al., 2009), which suggests that oral ATP itself is unlikely to be useful for improving glucose control. Whether a small molecular P2R agonist, delivered systemically, could replicate the benefits of eATP reported here will be important to determine. A better understanding of the purinergic regulation of glucose uptake, metabolism and cytokine release in conditions with compromised insulin and anabolic sensitivity is required to assess therapeutic potential of this system.

In summary, we show that extracellular ATP release does not contribute to reduced insulin sensitivity induced by palmitate in skeletal muscle myotubes but does increase glucose uptake and metabolism by a P2R-dependent mechanism. Importantly, the cytokine profile generated by ATP/ATPγS is similar to the signature generated by moderate and high intensity exercise, which has been shown to increases muscle and plasma ATP levels; increase skeletal muscle glucose uptake (Carey et al., 2006; Ikeda et al., 2016); increase circulating levels of IL-6 (Sprenger et al., 1992) and to decrease circulating MIF levels (Wahl et al., 2014). The potential for the exercise mimetic actions of ATP and P2R modulation in skeletal muscle to treat conditions such as T2D requires further study.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

AC designed and performed experiments, analyzed data and contributed to interpretation of data and writing of the manuscript. CB conceived the study, contributed to experimental design and wrote the manuscript. Both authors agree to be accountable for the content of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.567378/full#supplementary-material

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REVIEW
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Crosstalk Between Skeletal Muscle and Immune System: Which Roles Do IL-6 and Glutamine Play?

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The skeletal muscle was always seen from biomechanical and biochemical views. It is well-established that an active muscle brings many benefits for different body organs and tissues, including the immune system. Since the 1970s, many studies have shown the importance of regular exercise and physical activity in increasing the body's ability to fight opportunist infections, as well as a strategy to fight established diseases. This interaction was mainly attributed to the glutamine, a non-essential amino acid produced by the active skeletal muscle and primarily consumed by rapidly dividing cells, including lymphocytes and monocytes/macrophages, as their main source of energy. Therefore, these cells' function would be significantly improved by the presence of a bigger glutamine pool, facilitating phagocytosis, antigen-presentation, proliferative capacity, cytokine synthesis and release, among other functions. Despite its importance, glutamine is not the only molecule to connect these two tissues. The presence of cytokines is crucial for a proper immune system function. Many of them have well-established pro-inflammatory properties, while others are known for their anti-inflammatory role. Interleukin-6 (IL-6), however, has been in the center of many scientific discussions since it can act as pro- and anti-inflammatory cytokine depending on the tissue that releases it. Skeletal muscle is an essential source of IL-6 with anti-inflammatory properties, regulating the function of the immune cells after tissue injury and the healing process. Therefore, this review aims to discuss further the role of these four components (glutamine, and interleukin-6, and its interface with monocytes/macrophages, and lymphocytes) on the communication between the skeletal muscle and the immune system.

Keywords: skeletal muscle, immune system, glutamine, interleukin-6, lymphocytes, macrophages

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INTRODUCTION

For years, the skeletal muscle was seen from a biomechanical point of view as an organ responsible for producing movement thanks to the contraction of its fibers. Later, the importance of this organ from a biochemical point of view was discovered. It was observed that the skeletal muscle is a crucial energy-consumer tissue when active, consuming glucose and glycogen as essential energy

sources, but also consuming the energy accumulated in the adipose tissue, leading to lower body fat percentage, associated with many health and metabolic benefits.

Studies dating back to the 1970s, especially those focused on exercise, have shown the importance of physical activity to the immune system. It has been shown that regular physical activity is essential to increase the organism's ability to fight opportunist infections, despite an initial, transient exercise-induced immunosuppression (Ahlborg and Ahlborg, 1970; Calder et al., 2007).

For that to happen, a proper metabolic environment plays a pivotal role. The proper plasma concentration of glutamine mainly provides this environment. Glutamine was maybe the first well-established link between the immune system and the skeletal muscle. Under optimal conditions, there is a qualitative and quantitative impact on the immune cells and the muscle itself, improving its ability to secrete proteins known as myokines.

Leukocytes, in general, are sensitive cells affected by exercise. According to Pedersen and Hoffman-Goetz (2000), exercise can induce stress-like responses in the body, similar to those observed after major surgeries, trauma, severe burn, and sepsis. These physical-clinical stressors induce mechanical, metabolic, and hormonal responses to keep body homeostasis. As part of its process, the immune system is affected, with acute and chronic adaptations, leading to adjustments in the inflammatory response and the response of neutrophils, lymphocytes, and monocytes (Pedersen and Hoffman-Goetz, 2000).

Such cells are responsive to hormones, such as adrenaline and cortisol, as well as to cytokines, to keep their constant communication with the skeletal muscle. More recently, proteins similar to the cytokines have been discovered to be produced by the skeletal muscle itself. The so-called myokines, produced by the myocytes and released by muscle contraction during physical exercise, have autocrine, paracrine, and endocrine functions, and help perform the regulation of the immunometabolism thanks to their ability to induce significant metabolic, energetic and hormonal changes (Cannon, 2000; Pillon et al., 2013; Iizuka et al., 2014; Severinsen and Pedersen, 2020).

In this review, summarized in **Figure 1**, we will highlight the important roles of glutamine and interleukin-6 (IL-6), as well as lymphocytes and monocytes/macrophages in the complex communication between the skeletal muscle and the immune system.

GLUTAMINE

Glutamine is the most abundant free amino acid in the body. It plays a pivotal role in maintaining the function of several organs and cells, such as kidneys, intestines, liver, heart, neurons, leukocytes, and white adipose tissue (Curi et al., 2017; Cruzat et al., 2018). Its production by the skeletal muscle in healthy subjects classifies the glutamine as a non-essential amino acid, however, glutamine concentration varies according to the type of muscle fibers. Type 1 fibers or oxidative fibers can present up to three times more glutamine than type 2 (glycolytic) fibers since type 1 fibers present more glutamine synthetase and more ATP availability than the later (Cruzat and Tirapegui, 2009; Cruzat et al., 2018).

Glutamine may also be considered a conditionally essential amino acid for the amount produced under stressful conditions, such as severe burn, sepsis, infections, major surgeries, and intense exercise, may not be enough to maintain the proper function of the organs and cells previously mentioned (Curi et al., 2017; Soares et al., 2019).

Glutamine is synthesized mainly by the skeletal muscle in an ATP-dependent reaction mediated by glutamine synthetase (GS), which catalyzes it from glutamate and ammonia (glutaminase being the enzyme that catalyzes the reverse reaction, however, it is not found in the skeletal muscle). Glutamine is then released from the muscle and transported to the plasma by a bidirectional N^m transportation system affected by glucocorticoids and insulin levels (Walsh et al., 1998; dos Santos et al., 2009).

Glutamine levels increase after intense, short-term exercise and drop after intense, prolonged exercise (Walsh et al., 1998; dos Santos et al., 2009). Although the mechanisms are still under investigation, authors have proposed some mechanisms to explain this phenomenon: high demand by the liver and kidneys for glucose relying on gluconeogenesis to fulfill their demand; increase consumption of glutamine by the immune and other cells; impairment of the mechanisms that promote the release of glutamine by the muscle; and/or a decrease of glutamine synthesis by the muscle (Walsh et al., 1998; dos Santos et al., 2009). In order to shed some light on this subject, dos Santos et al. evaluated different aspects related to the glutamine metabolism: its plasma levels, its transport, GS activity, among others. They used 47 animals distributed in sedentary and trained groups, the later divided into two groups of animals sacrificed 1 h after the last exercise session, and the second sacrificed 24 h after the last exercise session. In possession of plasma and the soleus muscle, the authors observed that glutamine levels were lower in animals sacrificed 1 h after the last exercise session, with a concomitant increase in the corticosterone plasma levels and the GS activity, and lower ammonia levels in the muscle suggesting higher consumption of glutamine by other tissues, such as liver and kidneys. On the other hand, animals sacrificed 24 h after the last exercise session had similar glutamine levels to sedentary animals, with lower plasma levels of corticosterone, lower GS activity, and lower glutamine concentration in the muscle, supporting the lower restoration hypothesis (dos Santos et al., 2009).

Glutamine is an essential fuel for rapidly dividing cells, such as enterocytes, fibroblasts, and leukocytes because it is a precursor of peptides, proteins, nicotinamide adenine dinucleotide phosphate (NADPH), antioxidants, purines, and pyrimidines (Aledo, 2004; Curi et al., 2017). Glutamine also plays an important role regulating the heat shock proteins (HSP) and the reactive oxygen species (ROS), which depending on the intensity and duration of the exercise, can lead to muscle catabolism that contributes to reduce glutamine concentration (Cruzat and Tirapegui, 2009). Therefore, glutamine prepares the physiological environment for these cells' best function and performance.

When glutamine concentration lowers under one of the stressful conditions mentioned above, cells, such as

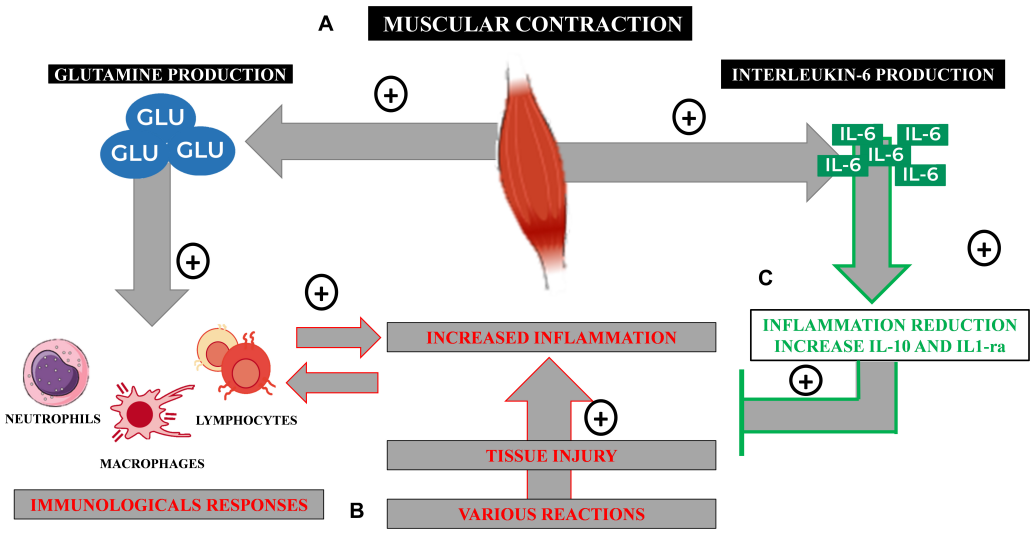

FIGURE 1 | Immunometabolic processes from the practice of physical exercises. **(A)** Glutamine is synthesized by the active skeletal muscle in an ATP-dependent reaction and released from it to the plasma by a bidirectional Nm transportation system. **(B)** Under infectious or inflammatory conditions that lead to tissue injury, an inflammatory reaction takes place activating immune cells, such as neutrophils, macrophages, and lymphocytes. These cells consume large amounts of glutamine to keep their function and immunological performance, including pro-inflammatory cytokines' synthesis, such as IL-6. **(C)** The skeletal muscle is also capable of producing myokines, such as IL-6, that, in this case, has an anti-inflammatory property, regulating inflammation and assisting on tissue healing processes.

lymphocytes, macrophages, and neutrophils, have their function and performance impaired due to the lack of their primary source of fuel. Consequently, it is observed lower neutrophils oxidative burst, a decline in some lymphocyte T populations, an impairment of T cell proliferative capacity, and lower macrophages' phagocytic ability. These conditions lead to immunosuppression, increasing the chances of a person developing infections, such as upper respiratory tract infections (Bassit et al., 2002; Rogeri and Rosa, 2005; Curi et al., 2017; Soares et al., 2019).

Although glutamine production happens primarily by active skeletal muscle, intense muscle contraction increases the demand for glutamine, which competes for the same fuel nutrient as lymphocytes and macrophages, forcing a modulation of these cells in favor of the musculature (Newsholme, 1994). A study with 11 healthy subjects showed that glutamine supplementation was able to increase the glutamine uptake by the skeletal muscle, however, it did not increase the intramuscular concentration of this amino acid, suggesting that there is either a simultaneous increase in the protein synthesis in the tissue or a limit to its accumulation in the muscle (Mittendorfer et al., 2001). Therefore, despite its production, the skeletal muscle also consumes glutamine lowering its availability for other tissues and cells.

During infection, the consumption of glutamine by immune cells is higher than glucose, since glutamine is necessary for T and B lymphocytes proliferation process, as well as for protein synthesis, production of interleukin-2 (IL- 2) and antibody synthesis (Cruzat et al., 2018). Therefore, glutamine metabolism plays a crucial role in lymphocyte activation, and its decline in plasma concentration after intense exercise has been observed (Keast et al., 1995). Also, low levels have been reported as a predictor of overtraining in athletes (Keast et al., 1995). However, the low availability of glutamine cannot be observed in every catabolic or ill patient, and not all individuals benefit from glutamine supplementation. In fact, there is not enough evidence in the literature showing that glutamine supplementation restores immune function after exercise (Keast et al., 1995) and the results of such studies remain controversial and seem to vary according to many factors, such as its form (free or dipeptide) or the association or not with other supplements (Cruzat et al., 2014). A recent study showed that athletes who undergo rapid weight loss for competition purposes, creating significant stress levels to their bodies, did not benefit from glutamine supplementation. The study showed that such athletes present an increased frequency of upper respiratory tract infections in spite of glutamine supplementation, similar to those who received placebo (Tritto et al., 2018).

Rogeri and Rosa (2005) studying spinal cord injured (SCI) people showed that in contrast to healthy subjects, people with that type of injury present a significant decrease in their plasma glutamine concentration. The authors also showed that the higher the injury, which leads to more spread out paralysis throughout the body, the lower the glutamine concentration, with a tendency to increase after a stress test in an adapted treadmill. Their findings suggested that glutamine concentration, and not only mechanical issues suffered by SCI people, is responsible for the high incidence of infection observed in this population when compared to healthy subjects. The authors also suggested that exercise may help restore glutamine concentration (Rogeri and Rosa, 2005).

Due to its importance to the immune system, glutamine became very popular and was consumed by many people as an attempt to improve their immune response. In the late 1980s and early 1990s, studies in animals have shown that

most of the glutamine orally consumed would not enter the bloodstream but instead remained in the intestinal lumen, being consumed by enterocytes (Newsholme, 1994). Therefore, studies have shown that a more efficient way to obtain positive results on the immune system is by consuming glutamine precursors, such as branched-chain amino acids (BCAA) (Bassit et al., 2000, 2002). Bassit et al. (2000, 2002) showed in two different studies that athletes that consumed placebo had a lower plasma glutamine concentration after their exercise session that was reverted by BCAA consumption with a consequent increase in the immune cells' proliferative capacity and cytokine modulation. Although most amino acids are metabolized in the liver, this organ possesses low BCAA aminotransferase activity, causing the BCAAs to be metabolized primarily in the skeletal muscle (Walsh et al., 1998).

Finally, glutamine degradation into glutamate, in a reaction catalyzed by glutaminase, as previously mentioned, provides an important precursor to glutathione synthesis, the most abundant non-protein thiol in the body. It acts as a powerful antioxidant, working in the xenobiotic detoxification, regulating essential cell functions such as proliferation and apoptosis, and acting upon the immune function and fibrogenesis. Therefore, the glutathione has a pivotal role in protecting the mitochondria against physiological and pathological stressors created by the ROS (Lu, 2013; Draganidis et al., 2016).

INTERLEUKIN-6

Interleukins are cytokines that trigger diverse immunomodulatory functions after changes in their physiological levels, which may induce pro-inflammatory, anti-inflammatory, or even both effects, according to the organism and/or the cell group in which they are synthesized (Brocker et al., 2010).

In this context, Interleukin-6 (IL-6) is the cytokine that shows the highest plasma elevations after acute physical exercise (Febbraio and Pedersen, 2002), with its plasma peak being directly influenced by the intensity (Leggate et al., 2010), the daily frequency (Ronsen et al., 2002), and/or the duration (Fischer, 2006) of the proposed exercise. It is currently proposed that the increase in IL-6, from muscle contraction, can trigger positive effects not only on muscle tissue but also on bone and mitochondrial health, and the control of low-grade chronic inflammation, through IL-6 anti-inflammatory effects in parallel with its performance in lipid oxidation (Fix et al., 2019; Wedell-Neergaard et al., 2019; Cornish et al., 2020).

In contrast to the acute elevations of IL-6 after physical exercise, the literature demonstrates that the improvement of physical conditioning in different populations is strongly associated with lower baseline (resting state) plasma values of IL-6 (Cesari et al., 2004; Colbert et al., 2004; Panagiotakos et al., 2005; Bruun et al., 2006). It suggests that increased levels of IL-6 in the absence of exercise may be directly related to a higher degree of physical inactivity and metabolic syndrome (Bruun et al., 2006; Fischer et al., 2007). This "contradictory action" of IL-6 occurs because this interleukin can be produced not only by the immune system cells, but also by different tissues such as adipocytes (Coppack, 2001; Lyngsø et al., 2002), and muscle fibers through the initial infiltration of macrophages in the muscle tissue (Tominaga et al., 2019) or by the subsequent production of this interleukin by myoblasts (Gallucci et al., 1998; Pillon et al., 2013), thus triggering systemic pro-inflammatory (Coppack, 2001) or anti-inflammatory (Gallucci et al., 1998; Pillon et al., 2013) effects, respectively.

Despite lacking recent original studies characterizing the biomolecular mechanisms behind the elevations of IL-6 in muscle fibers, some synergistic action between the infiltration of immune cells mediated by the practice of physical exercise in muscle tissue has been proposed as a determining factor for the regulation of muscle damage and inflammation (Gallucci et al., 1998; Kawanishi et al., 2016; Tominaga et al., 2019). In this context, the total plasma level of IL-6 can also be partially altered by immunological cells from the innate immune system (such as macrophages and neutrophils), as well as from the adaptive immune system (such as T and B cells) (Nielsen et al., 1996), depending on the training stimulus performed, ideally inducing transient pro-inflammatory effects, with a posterior anti-inflammatory response (Kawanishi et al., 2016).

From this perspective, the metabolic pathways, which can trigger substantial increases in IL-6 in muscle tissue, mediated by the regular practice of exercise, exert their effects locally on muscle cells, favoring more pronounced increases in IL-6 in the tissue, through a homodimer gp130Rb/IL-6Ra, which results in the activation of AMPK and/or phosphatidylinositol 3-kinase (PI3-kinase) (Pedersen and Fischer, 2007). In this sense, reduced concentration of muscle glycogen, previously or after the practice of exhaustive aerobic exercises, is considered an essential factor that favors the marked appearance of IL-6 in plasma via AMPK activation in myoblasts (Bartoccioni et al., 1994; Pedersen and Febbraio, 2008). This pathway can trigger more significant bioenergetic changes from the acute increase in IL-6. Also, it is proposed that this increase in plasma IL-6 levels may be partially influenced by the increased release of ionic calcium from the muscle sarcoplasmic reticulum, stimulating the activation of the nuclear factor of T cells (via calcineurin), which is present in the muscle (Bartoccioni et al., 1994; Holmes et al., 2004). In these two metabolic pathways, it has been shown that the produced IL-6 provides anti-inflammatory effects in the body, inhibiting, for example, endotoxin mediated by substantial increases in TNF-alpha levels in humans (Starkie et al., 2003; Keller et al., 2006; Hennigar et al., 2017), in addition to inducing the subsequent release of other cytokines with anti-inflammatory function [interleukin-1 receptor antagonist (IL-1ra) and interleukin-10 (IL-10)] (Hotamisligil et al., 1996).

Currently, these physiological actions were considered relevant to indicate that the increase in IL-6, mediated by physical exercise, can trigger positive reflexes on the individual's insulin sensitivity (Steensberg, 2003), in addition to the increase in lipid oxidation (Carey et al., 2006), without performing "undesirable" pro-inflammatory effects to the proper functioning of the immune energy metabolism. Also, increases in IL-6 with an anti-inflammatory characteristic have been the target of encouraging studies involving a possible therapeutic effect of

IL-6 in chronic diseases that establish a chronic environment of low-grade inflammation, such as arthritis rheumatoid (Carey et al., 2006), sarcopenia (Beyer et al., 2012) and even cancer (Daou, 2020). In this sense, although it is not completely clear, IL-6 seems to be involved in immune metabolic issues from its production in myocytes and immune cells, during and immediately after the exercise.

In a recent study, Wedell-Neergaard et al. (2019) investigated the peripheral effects of IL-6. Fifty-three subjects with high central adiposity performed 12 weeks of aerobic training (intensities ranging from 50 to 85% of VO2max) with or without the presence of an IL-6 receptor blocker (tocilizumab). In the study (Wedell-Neergaard et al., 2019), the group that performed the training sessions with the administration of tocilizumab showed significant lower reductions in visceral fat compared to the group trained without administration of tocilizumab, with no lean mass and subcutaneous fat tissue difference. As mentioned by the authors "as visceral adipose tissue was found to express more IL-6 receptors than subcutaneous adipose tissue, it is most likely that visceral adipose tissue is more sensitive and responsive to changes in IL-6 than subcutaneous adipose tissue" (Wedell-Neergaard et al., 2019), indicating that not only the increase but also the action of IL-6 are strictly related to the visceral fat reduction in humans. Since central adiposity is associated with an increase in low-grade chronic inflammation, regardless of BMI (Wedell-Neergaard et al., 2018), it is relevant that future studies aim to clarify the mechanisms by which the acute increase in IL-6, in the context of exercise, could impact the mobilization of body fat deposits as well as its relationship with the recruitment of specific immune cells between different populations.

LYMPHOCYTES

Like most tissues, skeletal muscle contains a resident population and additional infiltrate immune cells during pathophysiological conditions, such as reperfusion-induced contraction or injury, endotoxemia, or inflammatory myopathies, due to the action of cytokines or factors with attractive properties and activation (Pillon et al., 2013).

Many studies have shown that exercise induces a short period of leukocytosis followed by another period of leukopenia, when mainly T cells suffer a significant decrease in its population, creating an opportunity for opportunistic infections to occur. According to Nieman (1994), immunological changes would be accentuated as the intensity increases, theory postulated on his famous "J" curve to explain the relationship between exercise intensity and risk of upper respiratory tract infections (URTI) (Nieman, 1994).

Intensity and duration of physical effort would be determinant to the proliferative response of T lymphocytes (Shinkai et al., 1992), as observed by a more significant increase in plasma cytokine levels at high intensities (Berk et al., 1989; Keast et al., 1995). In response to IL-2 released during intense muscle contraction, more natural killer (NK) cells, monocytes, and B cells are attracted due to their prominent responsiveness than any other subpopulation (Pedersen and Hoffman-Goetz, 2000), causing the relative decline of TCD4++ cells percentage (Fry et al., 1992).

Lymphocytes concentration decrease in the post-exercise period has also been associated with an apoptosis mechanism induced by exercise (Navalta et al., 2007) and more observed with a gradual increase in intensity, reaching the maximum peak immediately after exhaustive exercise (100% VO_2max), in percentages of apoptosis around 22% (Steensberg et al., 2002). There are reports about 63% of lymphocytes apoptosis after high intensity (Mars et al., 1998). Some authors tend to associate the phenomenon to action from high levels of catecholamines (Navalta et al., 2007), able to decrease the concentration of lymphocyte glutathione and increase oxidative stress (Wang and Huang, 2005) and the production of ROS, in addition to increased fragmentation of DNA (Mooren et al., 2002). However, the mechanism responsible for post-exercise apoptosis remains to be elucidated by science. In this meantime, researchers debate whether exercise could contribute to the marked apoptosis of lymphocytes, and criticize studies based on different sampling time, lack of methodologies standardization, and some subsets lymphocytes absence (Simpson et al., 2007; Navalta et al., 2010).

Overload during exercise causes microtrauma of varying degrees in muscle tissue that are considered temporary and repairable damage by the immune system, activated immediately after the injury by cellular debris and leakage of the cellular content from damaged fibers. Muscle contraction itself increases calcium and pro-inflammatory cytokines release, such as tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β), which together sarcolemma lesion and eicosanoids derived release (Smith, 2004) from the constituents from arachidonic acid of cell membranes, attract neutrophils, monocytes, lymphocytes and other cells to the injured site generating acute inflammatory response (Smith, 2000) and initiating cleaning and indirectly signalizing diapedesis (Moldoveanu et al., 2001), that is, the influx of cells to the site, vasodilation regulation, chemotactic activity and increase in permeability of the vascular endothelium (Tidball, 2005).

Both innate and adaptive immune systems are activated after muscle injury. However, their cells are recruited in an orderly manner to make the environment more conducive to each phase of regeneration. In a first pro-inflammatory moment, debris is cleared, and satellite cells are activated. T cells are removed to the lymphatic system mediated by the action of cortisol (Deyhle and Hyldahl, 2018), perhaps to avoid the potential risk of self-recognition of intracellular debris by the adaptive system, explaining how acute exercise does not redistribute T and B cells in the circulation in the same extent as other cells of innate system. Additionally, lactate production or increased acidity may impact leukocyte redistribution, associated with a higher catecholaminergic response that may also play a role in modifying this cell redistribution (Freidenreich and Volek, 2012).

Macrophages phagocyte the undesirable elements produced by tissue damage (Tidball, 2005). At the same time, IL-6 and interleukin-8 (IL-8) secreted after the damage stimulate the signaling pathway that activates NADPH-oxidase in the process known as respiratory burst, culminating in the release of ROSs (Brickson et al., 2001), chemokines, prostaglandins, hormones

such as insulin-like growth factor and some cell growth-regulating cytokines, such as transforming growth factor beta-1 (TGF-β1), which activate fibroblasts to secrete collagen molecules for tissue regeneration, in addition to activating satellite cells for restructuring tissue (Pedersen et al., 1998). This acute inflammatory response must be very well regulated to preserve the integrity of adjacent cells and tissues, avoiding exacerbating damage by exaggerating ROS production (Tidball, 2005). The balance between the pro and anti-inflammatory actions of different cytokines, controlled by an intrinsic program of satellite cells or modulated by extrinsic cells, such as eosinophils and T cells, contributes to the complete regeneration of damaged tissue (Petersen and Pedersen, 2005; Schiaffino et al., 2017).

In a second moment, T cells are recruited to convert the environment into anti-inflammatory and allow the expansion and differentiation of satellite cells and maturation of newly formed microfibers. M1 macrophages attract them about 3 days after the injury starts. They become involved in repairing the skeletal muscle by secreting a variety of growth factors and cytokines that modulate the microenvironment of inflammation. Similar to macrophages, T cells secrete growth factors and cytokines such as TNF-α, interferon gamma (IFN-γ), IL-1β, interleukin-4 (IL-4), interleukin-12 (IL-12), interleukin-13 (IL-13), which modulate the microenvironment to make it more conducive to muscle regeneration, raising the hypothesis that the inflammatory environment could activate and improve the functions of satellite cells (Yang and Hu, 2018).

T regulatory (Treg) cells are important controllers of immune tolerance and accumulate a few days after the injury, attracted by interleukin-33 (IL-33) concentration, a nuclear cytokine released during cell necrosis or tissue damage (Nascimento et al., 2017). In addition to regulating the cells directly responsible for repairing injured muscle, Treg also acts directly on tissue regeneration through the proliferation of muscle satellite cells, releasing amphiregulin, the main autocrine growth factor for human keratinocyte culture and a well-known promoter of tissue healing and regeneration (Burzyn et al., 2013).

Tregs can control inflammation by restricting the immune responses of other cells, both modulation of CD4, CD8 [via the release of inhibitory cytokines such as IL-10, TGF-β, and interleukin-35 (IL-35)] and NK cells (Panduro et al., 2018), controlling the behavior of neutrophils.

Tregs promote environment conversion from pro to anti-inflammatory by releasing anti-inflammatory cytokines (for example, IL-4, IL-10, IL-13) that stimulate M1 (bactericidal and inflammatory) to M2 (immunomodulatory) macrophages phenotype exchange, apoptosis or inhibition of neutrophil inflammatory activity (Li et al., 2018).

MONOCYTES/MACROPHAGES

After a muscle injury, an inflammatory response very well organized begins, leading to activation and differentiation of a variety of tissue and immune cells, aiming to repair the injury, leading to a complete recovery of the skeletal muscle (Cohen and Mosser, 2013; Peake et al., 2017).

After tissue injury, specific molecules known as chemotactic mediators are released to the bloodstream attracting monocytes, circulating cells from the immune system responsible for initiating, with neutrophils, the inflammation process, and tissue repair (Contrepois et al., 2020). Monocytes are heterogeneous cells, exhibiting specific functions, and are differentiated by their size, immune receptor expression, and proliferative capacity (Taylor et al., 2005; Shi and Pamer, 2011). They can be classified in three subtypes based on their cluster of differentiation, CD14 and CD16 (Strauss-Ayali et al., 2007) in classic monocytes (CD14++/CD16-) with phagocytic function and that also express genes involved to angiogenesis, wound healing, and coagulation (Hallam and Hagemann, 2012; Yang et al., 2014); non-classical pro-inflammatory monocytes (CD14+/CD16++) responsible for patrolling the tissues (Strauss-Ayali et al., 2007); and intermediate monocytes CD14++/CD16+ or pro-inflammatory monocytes (Hallam and Hagemann, 2012; Yang et al., 2014).

When tissue damage happens, monocytes migrate to the injured area and attach themselves to the extracellular matrix. Some components of the matrix, such as fibrinogen and collagen, seem to stimulate macrophage phagocytosis and pro-inflammatory factors expression (Dort et al., 2019).

The acute inflammatory response after tissue damage begins with neutrophil (Schneider and Tiidus, 2007; Kawanishi et al., 2010), followed by macrophage infiltration. The later acquire particular features depending on the microenvironment they attach to Kosmac et al. (2018). Macrophages represent the biggest pool of cells recruited to the skeletal muscle after injury and play a unique role in regulating the inflammatory process and tissue repair (Wang et al., 2014). Therefore, the recovery of the damaged tissue depends on the macrophage presence and action (Perandini et al., 2018).

In an experimental study with rats, Dort et al. (2019) observed that monocytes expressing Ly6Chi (with phagocytic and pro-inflammatory properties) secrete pro-inflammatory cytokines that attract more neutrophils and monocytes to the site of injury. This pro-inflammatory environment lasts for 48 h after the tissue damage. After that period, monocytes Ly6Clo, responsible for tissue repair, become more predominant, reducing the inflammatory process (Dort et al., 2019).

Once monocytes become resident cells, they also express different phenotypes depending on their activation state (Lee et al., 2020). They co-express CD11b and CD206 and participate in tissue repair by secreting chemotactic factors, having low phagocytic property (Dort et al., 2019). According to their immune function, resident macrophages can be classified in M1 or M2 macrophages (Dort et al., 2019). M1, or classically activated macrophages, have an overall pro-inflammatory behavior, secreting different cytokines, such as TNF-α, interleukin-1 alfa (IL-1α), monocyte chemoattractant protein 1 (MCP-1), monocyte chemotactic protein 3 (MCP-3), macrophage inflammatory protein 2 (MIP-2), oncostatin M (OSM), and vascular endothelial growth factor (VEGF). They also express high inducible nitric oxide synthase (iNOS) activity with a consequent increase in the ROS. M2 macrophages can be divided into three subsets, each one depending on a specific polarization

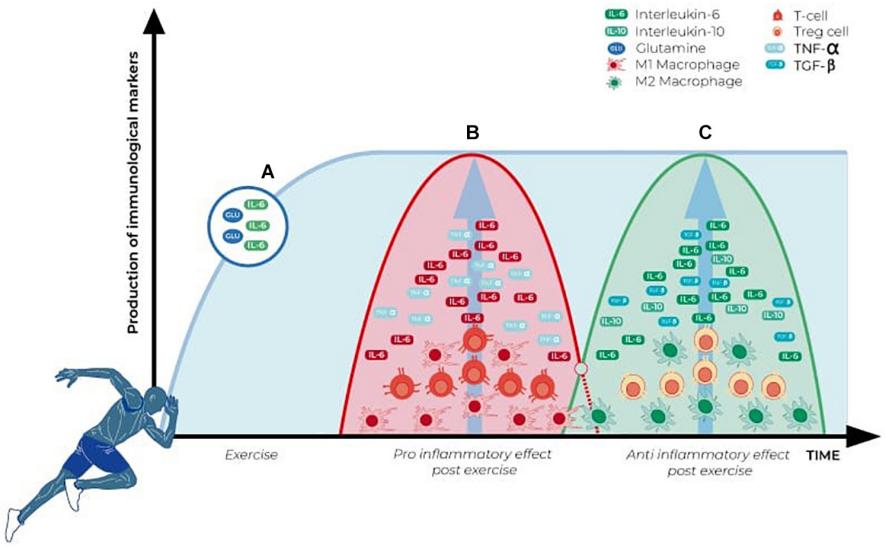

FIGURE 2 | Alteration in immune parameters associated with physical exercise and skeletal muscle activation. **(A)** Glutamine and IL-6 (myokine) levels increase during physical activity and remain elevated in the first moments after it ceases. **(B)** Pro-inflammatory wave initiated immediately after the exercise, with a significant increase in $CD4^+$ cells, M1 macrophages, and pro-inflammatory cytokines (TNF-α and IL-6). **(C)** Anti-inflammatory wave induced as a final response after the exercise-induced immune alterations, characterized by a significant increase in the anti-inflammatory cytokines (TGF-β, IL-10), myokines (IL-6), and activation of T regulatory cells and M2 macrophages.

signal. IL-4 and IL-13 exposure activates M2a macrophages, while M2b polarization happens through Il-1 receptor ligands, and M2c polarization is promoted by IL-10 and glucocorticoids (Dort et al., 2019; Lee et al., 2020). M2 macrophages are responsible for regulating the tissue repair process (Dort et al., 2019; Wang et al., 2014). In fact, M1 and M2 act in a perfect balance and together are responsible for the skeletal muscle homeostasis (Lee et al., 2020).

Lemos et al. (2015) showed that skeletal muscle resident M1 and M2 macrophage-produced cytokines modulate the extracellular matrix production through the fibro/adipogenic progenitor cells (FAPs). It has been shown that the production of extracellular matrix components by the FAPs is regulated by TNF-α and by the TGF-β1) secreted, respectively, by M1 and M2 macrophages. The kinetics between M1 and M2 macrophages after a skeletal muscle injury promotes FAPs apoptosis, avoiding an excessive extracellular matrix deposition on the tissue, and an inefficient regeneration process (Mann et al., 2011; Lemos et al., 2015). Based on these findings, FAPs and macrophages were characterized as part of the cells associated with a favorable microenvironment responsible for the activation and differentiation of satellite cells during the skeletal muscle repair process (Jonsdottir et al., 2000; Mashinchian et al., 2018).

After the skeletal muscle injury, an increase in the number of FAPs for the first 1–3 days starts, and it reduces between days 4 and 7 after injury (Lemos et al., 2015). This initial increase on the FAPs is essential for the production of the extracellular matrix components in order to stabilize the tissue, acting as a scaffolding for new fibers, being used by the satellite cells as a basal membrane to assure that the myofibers will remain aligned (Chen and Li, 2009; Mann et al., 2011; Lemos et al., 2015; Munoz-Canoves and Serrano, 2015). This process must be tightly regulated, and the FAPs decline is essential to prevent excessive extracellular matrix deposition, impairing tissue regeneration (Mann et al., 2011).

FAPs kinetics is modulated by cytokines produced and released by both pro- and anti-inflammatory macrophages. A study showed that TNF-α leads to a significant decrease in FAPs after a skeletal muscle injury and that the primary source of this cytokine is the pro-inflammatory macrophages, showing the importance of this joint work between FAPs and macrophages to avoid excessive extracellular matrix deposition (Lemos et al., 2015). These findings were corroborated by studies that attempted to treat pulmonary fibrosis with an anti-TNF monoclonal antibody, which caused pathological accumulation of extracellular matrix (63.64). Plus, pro-inflammatory macrophages increased is followed by anti-inflammatory macrophages during skeletal muscle tissue repair, increasing the expression of TGF-β, which blocks FAPs' TNF-α-induced apoptosis (Arnold et al., 2007).

While acute inflammatory response is associated to a proper skeletal muscle tissue repair and regeneration, chronic, non-decisive inflammation, such as those observed in pathological conditions like idiopathic inflammatory myopathies, dystrophies, and obesity are associated to impaired satellite, immune and FAP cells function, leading to increased fibrosis and weak muscle regeneration (Keller et al., 2003; Villalta et al., 2009; Kawanishi et al., 2010; Mann et al., 2011; Kong et al., 2013; Lemos et al., 2015). A consistent imbalance between pro- and anti-inflammatory macrophages in the skeletal muscle is associated with impaired satellite cell differentiation and activation (Li et al., 2003; Villalta et al., 2009). Also, chronic inflammation leads to an excess of cytokines responsible for

extracellular matrix production (Perandini et al., 2018), causing pro-fibrotic components accumulation, and therefore a non-favorable environment for proper muscle tissue repair (Villalta et al., 2009; Lemos et al., 2015).

Therapies aiming to reduce inflammation and muscle fibrosis have been developed with both beneficial and side effects (Li et al., 2003; Andreetta et al., 2006; Villalta et al., 2009). In an experimental model, it was observed that TGF-β inhibition reduces connective tissue and fibrosis in mice diaphragm but is followed by an increased inflammatory process (Andreetta et al., 2006). Also, some therapies attempted so far cause an imbalance between M1 and M2 cells, preventing the proper establishment of an environment that would allow satellite and other cells involved in the regeneration process to respond optimally (Li et al., 2003; Villalta et al., 2009).

Experimental studies showed that exercise could alter resident macrophages' phagocytosis, chemotaxis, and antigen presentation capacity abilities, indicating that physical activity can affect these cells function and phenotype (Kawanishi et al., 2010; Walsh et al., 2011).

A recent study showed that resident macrophages are capable of self-regeneration, are kept virtually the same up to adulthood, and respond to small attacks without monocyte infiltration (Davies et al., 2013). The skeletal muscle in response to exercise secretes protons, lactate, ATP, and other factors capable of directly activate macrophages and change their phenotype in response to stimuli (Jonsdottir et al., 2000; Keller et al., 2003; Li et al., 2003). For example, ATP increases pro-inflammatory cytokine release, promoting the expression of more M2 cells, while protons will increase endocytosis and IL-10 secretion by macrophages (Cohen and Mosser, 2013; Cohen et al., 2013; Kong et al., 2013).

Leung et al. (2016) showed a significant increase in the proportion between classic and regulatory macrophages after long-term exercise, suggesting that exercise can help the cells to transition between a pro-inflammatory to an anti-inflammatory state, or even create a mixed phenotype with characteristics both pro- and anti-inflammatory, which could be a protective factor against chronic pain after long or intense exercise sessions (Leung et al., 2016).

CONCLUSION

The communication between the skeletal muscle and the immune system happens in many different ways and involves different aspects. Glutamine, a non-essential amino acid, seems to be strongly present in this communication. It is produced by the skeletal muscle, and is used as an energy source by leukocytes, mainly monocytes, and lymphocytes, but also is consumed by the muscle under intense contraction (Keast et al., 1995). In fact, its plasma level has been established as a marker for exercise severity (Keast et al., 1995). Studies have shown the importance of a healthy, in constant contraction skeletal muscle to keep glutamine at optimal levels to assist the immune response (Rogeri and Rosa, 2005). In some conditions, glutamine may not be adequately produced by the muscle, turning it into a conditionally essential amino acid (Curi et al., 2017; Cruzat et al., 2018). When glutamine concentration lowers under stressful conditions, cells such as lymphocytes, macrophages, and neutrophils have their function and performance impaired due to the lack of their primary source of fuel (Bassit et al., 2002; Rogeri and Rosa, 2005; Curi et al., 2017; Soares et al., 2019). A condition that can affect the immune system response due to the lack of glutamine is intense muscle contraction. In this situation, the glutamine produced is used by the muscle itself, turning the organ a competitor with the immune system for this critical substrate (Newsholme, 1994).

IL-6 also plays an essential role in this communication and is the interleukin that shows higher plasma levels after physical exercise (Febbraio and Pedersen, 2002). It can be produced both by the immune system and the skeletal muscle, with pro- and anti-inflammatory properties, respectively (Nielsen et al., 1996). This unique characteristic is important to modulate how immune cells will behave during tissue healing and repair.

Even under less intense exercise, the skeletal muscle suffers microlesions, and an inflammatory response takes place to solve it (Hedayatpour and Falla, 2015; Hody et al., 2019). This inflammatory response attracts immune cells from the circulation, while tissue-resident cells are activated. As **Figure 2** shows, this response happens in distinctive phases. At first, there is an elevation of glutamine and IL-6 to prepare the environment and provide substrates and chemotactic factors for the immune cells. In response to this microinjury, T cells are recruited while M1 resident macrophages are activated, initiating a first, pro-inflammatory wave (Yang and Hu, 2018). Lymphocytes behave in a biphasic pattern: the lymphocytosis observed right after the injury is followed by lymphocytopenia (Toft et al., 1992; Tidball, 2005; Freidenreich and Volek, 2012). However, these findings are mostly related to CD4+ lymphocytes. What happens is the removal of CD4+ cells from the area and an increase in T regulatory cells. These cells act with M2 macrophage cells from the tissue to control the inflammatory response, promoting a more efficient tissue repair, avoiding extracellular matrix excess and fibrosis (Mann et al., 2011; Lemos et al., 2015; Mashinchian et al., 2018; Yang and Hu, 2018).

Therefore, the communication between the skeletal muscle and the immune system seems to be very intense, finely tuned, and dependent on many different factors, such as the ones described above. The tight balance among them provides a proper environment not only for the skeletal muscle repair but also to improve immune system function and responsiveness.

AUTHOR CONTRIBUTIONS

PR and AL conceived the present idea. SG, GM, LC, CA, RL, and MK developed the theory. GM created the image. All authors discussed it and contributed to the final manuscript.

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Skeletal Muscle Immunometabolism in Women With Polycystic Ovary Syndrome: A Meta-Analysis

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Polycystic ovary syndrome (PCOS) is an endocrine and metabolic disorder affecting up to 15% of women at reproductive age. The main features of PCOS are hyperandrogenism and irregular menstrual cycles together with metabolic dysfunctions including hyperinsulinemia and insulin resistance and a 4-fold increased risk of developing type 2 diabetes. Despite the high prevalence the pathophysiology of the syndrome is unclear. Insulin resistance in women with PCOS likely affect the skeletal muscle and recently it was demonstrated that changes in DNA methylation affects the gene expression in skeletal muscle that in part can explain their metabolic abnormalities. The objective of this work was to combine gene expression array data from different datasets to improve statistical power and thereby identify novel biomarkers that can be further explored. In this narrative review, we performed a meta-analysis of skeletal muscle arrays available from Gene Expression Omnibus and from publications. The eligibility criteria were published articles in English, and baseline (no treatment) skeletal muscle samples from women with PCOS and controls. The R package Metafor was used for integration of the datasets. One hundred and fourteen unique transcripts were differentially expressed in skeletal muscle from women with PCOS vs. controls ($q < 0.05$), 87% of these transcripts have not been previously identified as altered in PCOS muscle. *ING2*, *CDKAL1,* and *AKTIP* had the largest differential increase in expression, and *TSHZ2, FKBP2*, and *OCEL1* had the largest decrease in expression. Two genes, *IRX3* and *CDKAL1* were consistently upregulated ($q < 0.05$) in the individual analyses and meta-analysis. Based on the meta-analysis, we identified several dysregulated immunometabolic pathways as a part of the molecular mechanisms of insulin resistance in the skeletal muscle of women with PCOS. The transcriptomic data need to be verified by functional analyses as well as proteomics to advance our understanding of PCOS specific insulin resistance in skeletal muscle.

Keywords: PCOS, transcriptomics, gene expression, immunometabolism, meta-analysis, skeletal muscle

INTRODUCTION

Polycystic ovary syndrome (PCOS) is a common endocrine disorder among women of reproductive age, which is characterized by biochemical or clinical hyperandrogenism, irregular cyclicity and polycystic ovarian morphology (Norman et al., 2007; Teede et al., 2018). Metabolic dysfunction is evident in women with PCOS, and manifests as impaired glucose homeostasis, dyslipidemia and abdominal obesity (Azziz et al., 2016). Women with PCOS have epigenetic and transcriptional changes in skeletal muscle that, in part, can explain the metabolic abnormalities seen in these women (Skov et al., 2007; Nilsson et al., 2018). In addition, defects in early insulin signaling in combination with fibrosis in the skeletal muscle, likely contribute to insulin resistance in women with PCOS (Stepto et al., 2020).

There is an increasing body of publications supporting a role for both innate and adaptive immunity in response to changes in metabolic status. This new field of immunometabolism builds on evidence for activation of immune-derived signals in metabolically relevant tissues and explores how immune cells support tissues to adapt to environmental challenges (Man et al., 2017). Adipose tissue is one of the most explored tissues in the field, and the expression and activation of various immune cell types and anti-inflammatory cytokines have been studied in both lean and obese states (Ferrante, 2013; Man et al., 2017). Skeletal muscle is another key metabolically active organ considering that it is the most effective organ for insulin-stimulated glucose uptake in the body (∼80%) (Thiebaud et al., 1982). It also plays an important role in the development and progression of type 2 diabetes (Petersen and Shulman, 2018). However, there is scarce knowledge on how immune cells support the metabolic function in the skeletal muscle and on the role of inflammation in modulating skeletal muscle metabolism. The possible impact of immune cells on whole body glucose uptake and insulin sensitivity is a relatively recent appreciation.

Insulin resistance is a central feature of PCOS, with 30–40% of women with the syndrome having glucose intolerance, and 10% of them develop type 2 diabetes before the age of 40 (Legro et al., 1999; Rubin et al., 2017). Clinical studies exploring the molecular pathways of PCOS-insulin resistance in the skeletal muscle are limited. We have previously performed a wide-scale transcriptomic analysis in the skeletal muscle of women with PCOS and reported that many enriched pathways were involved in immune function or immune diseases (Nilsson et al., 2018). Due to the limited sample size of our study and the need to better understand the skeletal muscle metabolism in PCOS, we aim here to perform an integrated meta-analysis of the three available gene expression arrays in skeletal muscle of overweight/obese women with impaired glucose homeostasis. We further explore whether immunometabolism pathways are dysregulated in the skeletal muscle of these women.

METHODS

Selection of Studies

We collected array studies including skeletal muscle from women with and without PCOS published between January 1999 and August 21, 2020, by performing a computerized search using PubMed, Omnibus GEO and Array Express. No review protocol exists but the following key words were used: human, skeletal muscle, polycystic ovary syndrome, PCOS, array, and gene expression. The eligibility criteria were; published articles, published in English, and baseline samples (no treatment). Data was collected from the GEO database (Skov et al., 2007, 2008) and from our own publication (Nilsson et al., 2018). The analysis is reported according to the PRISMA checklist (Moher et al., 2009).

Meta-Analysis

The datasets were collected from two different microarray platforms, namely Affymetrix and Illumina. Affymetrix data was downloaded from NCBI GEO (GSE8157 and GSE6798), and normalized with methods described in Skov et al. (2007). The CEL-files and additional files were downloaded using the GEOquery R package. In order to check which samples were present in both GSE8157 and GSE6798, since some samples was used in both studies (Skov et al., 2007, 2008) but with different names, we used the R package DupChecker to compare the CEL-files. The Illumina data was background corrected and normalized using NormExp method in the R package limma (Shi et al., 2010). A fixed-effect meta-analysis model, with inverse-variance method, was used to estimate the possible associations between gene expression and PCOS. The Q statistic from the meta-analysis was used to identify the presence of heterogeneity. To account for multiple testing and control for false positives, the p-values from the meta-analysis were adjusted, using the Benjamini-Hochberg procedure. The robust genes, showing statistically significant association with PCOS, were identified as genes with false discovery rate (FDR) $q < 0.05$, and are presented as effect size. A heat map for all significant genes ($q < 0.05$) and a forest plot for the two most robust genes were produced. All analyses and plots were performed using the R statistical programming software [Version 4.0.2]. The meta-analysis was conducted using the rma() function in the Metafor package (Viechtbauer, 2010), the heatmap was produced using the heatmap.2() function in the gplot package and the FDR was estimated using the stats package.

Pathway Analyses

We applied gene set enrichment analysis (GSEA. https://www.gsea-msigdb.org/gsea/index.jsp) to the expression array data using Broad Institute Gene sets database. All transcripts were used and ranked according to the t statistics in a t-test. The GSEA considered pathways with 5–5,000 transcripts. Finally, we performed a Gene Ontology (GO) enrichment analysis using the DAVID web resource (Huang da et al., 2009) to identify those biological functions and processes that are shared by the differentially expressed genes ($q < 0.05$). Gene groups were subjected to Functional Annotation Clustering using the GOTERM_BP_FAT and KEGG_PATHWAY categories, and annotation clusters with enrichment score at least 1.3, corresponding to $P < 0.05$ (Huang da et al., 2009), were considered enriched.

TABLE 1 | Clinical characteristics of the patients in the 3 cohorts included in the meta-analysis.

Characteristic	Skov et al. (2007) Control/PCOS	Skov et al. (2008) Control/PCOS	Nilsson et al. (2018) Control/PCOS
Total subjects	13/16	13/10	14/17
Included subjects	13/16	0/3	14/17
Age (years)	34.7 ± 2.0/30.8 ± 1.8	34.7 ± 2.0/30.3 ± 2.1	29.7 ± 5.85/31.2 ± 5.4
BMI (kg/m^2)	34.0 ± 1.8/34.1 ± 1.1	34.0 ± 1.8/33.2 ± 0.9	30.2 ± 3.6/31.3 ± 4.3
Insulin (pmol/L)	43 ± 4/116 ± 16*	51 ± 6/125 ± 23*	63 ± 27/89 ± 55
Glucose (mmol/L)	5.5 ± 0.1/5.5 ± 0.1	5.6 ± 0.1/5.9 ± 0.2	5.1 ± 0.4/4.9 ± 0.3
Free testosterone (nmol/L)	0.021 ± 0.000/0.059 ± 0.010*	0.025 ± 0.003/0.053 ± 0.009*	0.015 ± 0.005/0.029 ± 0.019*
LH/FSH ratio	0.68 ± 0.06/1.55 ± 0.17*	No data	1.18 ± 0.84/1.80 ± 1.13

*In total, 27 controls and 36 women with PCOS were included. *P < 0.05 PCOS vs. control subjects in each cohort. Data presented as mean ± SEM in Skov et al. (2007, 2008), and as mean ± SD in Nilsson et al. (2018).*

RESULTS

Meta-Analysis of mRNA Expression in Skeletal Muscle From Women With PCOS and Controls

A total of 24 studies were identified, 12 were assessed for eligibility, and three studies were included in the meta-analysis as they fulfilled the eligibility criteria; published articles, published in English, and baseline samples (no treatment) (Skov et al., 2007, 2008; Nilsson et al., 2018). A PRISMA flow diagram is included in **Supplementary Figure 1** (Prisma checklist in **Supplementary Table 1**). Only 3 samples in GSE8157 were exclusive for this study, while the others were also included in GSE6798. This generated a total number of 27 controls and 36 PCOS cases in the meta-analysis. **Table 1** shows the most relevant clinical characteristics of the patients from each cohort. All women were over-weight or obese (mean BMI > 30 kg/m^2), and premenopausal. Women with PCOS were hyperandrogenic with metabolic aberration and insulin resistance. PCOS was diagnosed according to 2 criteria; irregular periods with cycle length > 35 days, and biochemical or clinical signs of hyperandrogenism in Skov et al. (2007, 2008), and according to Rotterdam criteria in Nilsson et al. (2018) fulfilling 2 of 3 criteria; ultrasound-verified polycystic ovaries, oligomenorrhea/amenorrhea, and/or clinical signs of hyperandrogenism.

In total, of the 8,351 analyzed transcripts, 1,228 were differentially expressed in skeletal muscle from women with PCOS vs. controls ($P < 0.05$). After correction for multiple testing by FDR (5%, $q < 0.05$), 114 unique transcripts remained significant (**Figure 1A**, **Table 2**). In total, 82% of the transcripts were upregulated and 18% were downregulated in women with PCOS. The three genes with the largest differential increase in expression were *ING2*, *CDKAL1,* and *AKTIP,* and the three genes with the largest differential decrease in expression were *TSHZ2, FKBP2*, and *OCEL1*. Ninety-nine of the 114 identified transcripts have not been previously shown to be altered in skeletal muscle arrays from women with PCOS (Skov et al., 2007, 2008; Nilsson et al., 2018) (marked in bold in **Table 2**).

In Nilsson et al. (2018), we have previously investigated the overlap between the differentially expressed genes identified in that study ($p < 0.05$) and those found in Skov et al. (2007) ($q < 0.1$), and found four transcripts (i.e., *RAPH1, INO80D, PPIE*, and *AKTIP*) that were upregulated in both studies. Two of these genes, *RAPH1* and *AKTIP* were significant at $q < 0.05$ (effect size 1.16 and 1.17, respectively) and *PPIE* at $q < 0.1$ (effect size 0.86) in the integrated meta-analysis, while *INO80D* was not significantly altered. The meta-analysis findings were also compared with those obtained by individual analyses in both datasets (**Supplementary Table 2**) to evaluate bias and reproducibility across the microarray studies. As a result, 34 genes identified in the Illumina data set were also highlighted by our meta-analysis, whereas 20 genes were shared with the Affymetrix data set (**Figure 1B**). Two of these genes, *IRX3* (effect size 1.09) and *CDKAL1* (effect size 1.27) were consistently upregulated ($q < 0.05$), by the three studies (Affymetrix, Illumina and integrated meta-analysis) (**Figure 1C**).

Gene Set Enrichment Analysis and Pathway Analysis

Next, we tested whether sets of biologically-related genes were altered in women with PCOS compared with controls. According to the GSEA, there were significant ($q < 0.05$) gene expression alterations in 9 downregulated pathways in women with PCOS (**Table 3**). The majority of these downregulated pathways are involved in inflammatory/immune response while myogenesis and MYC signaling, a transcription factor for growth-related genes for glycolysis and mitochondrial metabolism in T cells (Wang et al., 2011), were upregulated ($p < 0.05$). Finally, GO analysis including the 114 differently expressed transcripts ($q < 0.05$) showed 205 enriched signaling pathways (**Figure 2A**, **Supplementary Table 3**). Genes involved in metabolic and carbohydrate biosynthetic processes, positive regulation of I-kappaB kinase/NF-kappaB signaling, and negative regulation of cell proliferation and positive regulation of cell death appeared in the enriched pathways (**Figure 2B**). Muscle system processes and calcium ion transport were also enriched together with the androgen receptor signaling pathway (**Figure 2B**, **Supplementary Table 3**). All three genes in the androgen receptor signaling pathway; *RAN, RB1,* and *CTNNB1*, were

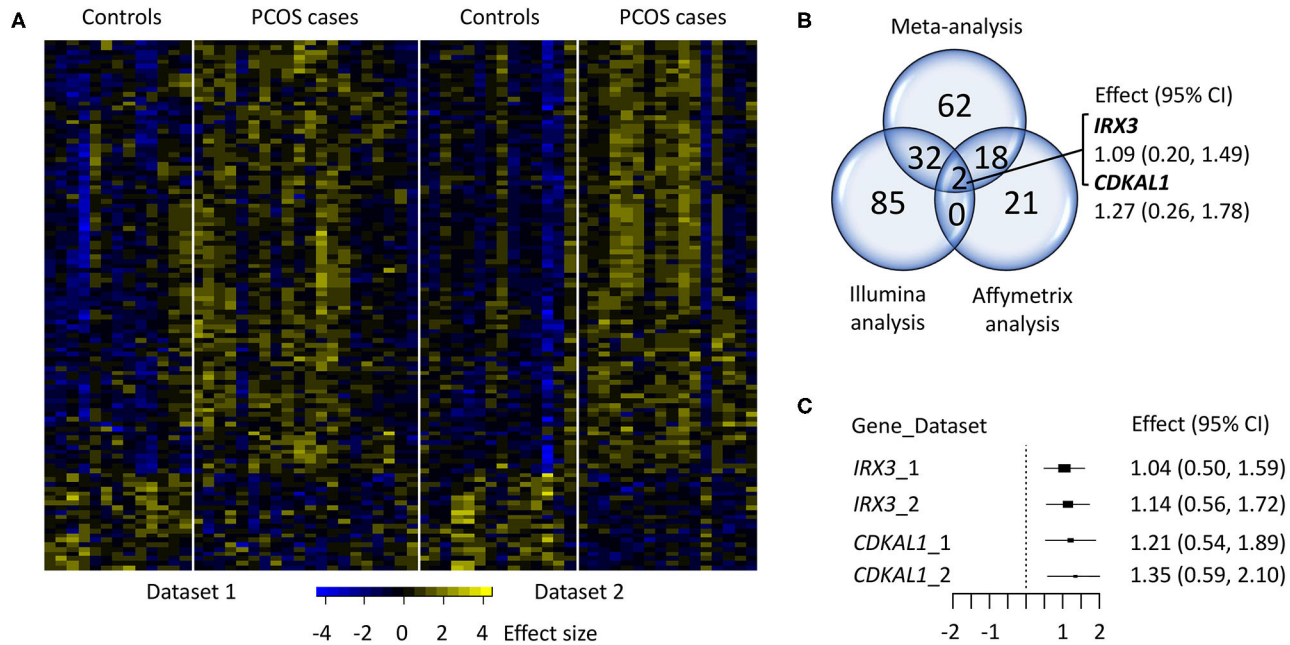

FIGURE 1 | Comparison of individual analysis in dataset 1 (Skov et al., 2007, 2008) and 2 (Nilsson et al., 2018). **(A)** Heat map of the 114 differentially expressed genes ($q < 0.05$), **(B)** Venn diagram of the individual analysis; Illumina, Affymetrix, and integrated meta-analysis ($q < 0.05$). **(C)** Forest plot of the effect size of the two genes, *IRX3,* and *CDKAL1,* consistently upregulated in the three studies.

significantly upregulated in women with PCOS compared to controls ($q < 0.05$, **Table 2**).

DISCUSSION

Taking an integrated meta-analysis approach, this study showed an overall downregulation of transcripts involved in the immune system and several alterations in metabolic pathways in skeletal muscle of women with PCOS. Previous data suggests that insulin signaling defects in skeletal muscle partly account for the insulin resistance in obese women with PCOS. However, our data indicate that additional molecular mechanisms are involved in the PCOS-insulin resistance in skeletal muscle. Fibrosis might be one key player (Stepto et al., 2020), together with immunometabolic alterations as supported by our data.

Fibrosis in skeletal muscle can impair muscle function and affect muscle fiber regeneration after injury (Delaney et al., 2017; Mahdy, 2019). Tissue fibrosis is often initiated and maintained through TGF-beta signaling and has been suggested to be associated with insulin resistance and steatosis in PCOS (Petta et al., 2017; Stepto et al., 2019). This association is supported by a recent paper, investigating TGF-beta ligand induced fibrosis in obese women with PCOS (Stepto et al., 2020). Moreover, we have previously shown an enrichment of extracellular matrix signaling pathways and a decreased collagen gene expression in our previous study (Nilsson et al., 2018), supporting a link between dysregulated TGF-beta signaling and extracellular matrix deposition with insulin resistance in PCOS. Surprisingly, fibrosis, extracellular matrix-, or TGF-beta signaling pathways were not enriched in women with PCOS in this meta-analysis. However, the most upregulated gene in PCOS muscle, inhibitor of growth family member 2 (*ING2*), promotes TGF-beta-induced transcription and cellular responses (Sarker et al., 2008), which leave the issue open for further investigation.

We have previously shown that the majority of the significant gene expression pathways (GSEA) in women with PCOS were involved in immune responses or were related to immune diseases (Nilsson et al., 2018). A distinct pattern was a downregulation of human leukocyte antigen (HLA) genes in women with PCOS. This finding was not validated in this meta-analysis, although two HLA genes were downregulated ($p < 0.05$), but they did not survive FDR < 5%. However, here we show a dysregulated expression of many genes involved in immune pathways, including a downregulation of gene sets associated with inflammatory response, interferon alpha and gamma response and the proinflammatory cytokines interleukin-2, interleukin-6 and TNF-alpha. Among the dysregulated genes, *ADK* and *BST2* are thought to play a role in the immune system, identified as having anti-inflammatory and anti-viral actions, respectively (Douglas et al., 2010; Boison, 2013). *BST2* was one of the top downregulated genes, and is known to be an interferon-inducible gene, and to play a role in innate immunity (Douglas et al., 2010). *FKBP2* was also one of the top downregulated genes, which plays a role in immunoregulation by binding the immunosuppressive compound rapamycin that inhibits mTOR signaling (Hendrickson et al., 1993). This is in line with

TABLE 2 | Differentially expressed transcripts in skeletal muscle from women with PCOS vs. controls in this meta-analysis ($q < 0.05$).

Gene	ES	q	Gene	ES	q	Gene	ES	q
ING2	1.32	0.002	***MYLK4***	0.93	0.004	***AES***	0.78	0.050
CDKAL1	1.27	0.002	***USP53***	0.93	0.017	***INPP5A***	0.77	0.047
AKTIP	1.17	0.020	***NFIA***	0.92	0.033	***MOCS2***	0.75	0.030
RAPH1	1.16	0.004	***STXBP3***	0.92	0.019	***UBR3***	0.75	0.030
UBC	1.15	0.040	***RRM2B***	0.92	0.013	***ANXA7***	0.75	0.044
ADK	1.13	0.004	***PSMC4***	0.92	0.050	***NEDD1***	0.74	0.031
HFE2	1.13	0.004	***PPP1R3B***	0.92	0.009	***COL4A3***	0.74	0.038
CSPP1	1.11	0.007	***RBM24***	0.91	0.017	***FAM129A***	0.74	0.033
SCP2	1.11	0.005	***PLCL1***	0.91	0.017	***MSRB3***	0.74	0.049
MAPKAP1	1.11	0.004	***HIST1H2AC***	0.90	0.011	***C1ORF43***	0.74	0.047
CACNB1	1.10	0.013	***VCL***	0.89	0.042	***ENSA***	0.74	0.030
ABLIM1	1.10	0.005	***KRR1***	0.89	0.042	***CLK1***	0.73	0.047
IRX3	1.09	0.001	***NGDN***	0.89	0.042	***BPGM***	0.73	0.050
UNC13B	1.09	0.012	***FBXW7***	0.88	0.010	***FBXO32***	0.72	0.047
SHISA2	1.08	0.002	***MCL1***	0.88	0.022	***CALML6***	0.70	0.017
HSF2	1.08	0.012	***PGK1***	0.87	0.023	***MLF1***	0.69	0.048
DUSP13	1.07	0.026	***PHKG1***	0.87	0.009	***LRRC3B***	0.68	0.030
IREB2	1.07	0.005	*PDE4DIP*	0.87	0.017	***LPL***	−0.71	0.046
SYNC	1.05	0.005	***EGF***	0.86	0.030	***HMOX1***	−0.76	0.038
ADPRHL1	1.04	0.008	***GRB14***	0.86	0.007	***ADM***	−0.77	0.049
ATP2A1	1.02	0.002	***CTNNB1***	0.85	0.030	***UCP2***	−0.78	0.026
MORC3	1.01	0.004	***SERPINE2***	0.85	0.019	***MT2A***	−0.81	0.030
RCN2	1.01	0.017	***FAM184B***	0.85	0.038	***SKAP2***	−0.81	0.030
ATP2C1	1.00	0.005	***TSPAN3***	0.85	0.018	***HSPB6***	−0.82	0.030
HOMER1	1.00	0.007	***CHAF1B***	0.85	0.031	***MT1E***	−0.85	0.031
OAT	1.00	0.008	***PTBP2***	0.84	0.033	***PC***	−0.86	0.033
MSTN	0.99	0.002	*PITX2*	0.84	0.045	***ATOH8***	−0.89	0.030
CTBP1	0.98	0.010	***ARMC8***	0.84	0.049	*NBPF20*	−0.89	0.045
ATP5G2	0.97	0.030	***NCKAP1***	0.83	0.012	***SNX21***	−0.89	0.033
EGFLAM	0.96	0.019	***PDZRN3***	0.83	0.035	***AGPAT9***	−0.89	0.030
TSC22D3	0.96	0.030	***ACVR1***	0.83	0.021	***LDHB***	−0.92	0.008
TMEM182	0.96	0.017	***TRDN***	0.83	0.013	***IFITM3***	−0.93	0.046
PRR16	0.95	0.004	***USP46***	0.81	0.047	*THY1*	−0.95	0.017
H2AFY	0.95	0.030	***GPD2***	0.81	0.043	***RSPO3***	−0.96	0.012
FRMD6	0.95	0.009	***RAN***	0.81	0.040	***BST2***	−1.08	0.016
CLTCL1	0.93	0.030	***ALDOA***	0.79	0.030	***OCEL1***	−1.15	0.013
DCAF6	0.93	0.042	***RB1***	0.79	0.041	*FKBP2*	−1.26	0.013
ZBTB16	0.93	0.020	***PPTC7***	0.79	0.042	***TSHZ2***	−1.29	0.006

Transcripts not previously shown to be altered in PCOS muscle arrays (Skov et al., 2007, 2008; Nilsson et al., 2018) are marked in bold. ES, Enrichment score.

the downregulation of enriched gene sets associated with interferon alpha and gamma responses. Looking into the most upregulated genes, *ADK* is a phosphotransferase that converts the purine ribonucleotide adenosine into 5′-adenosine-monophosphate (Boison, 2013). This reaction controls largely the adenosine tone, and consequently affects a wide range of functions. One of the functions that ADK and adenosine exert, is the regulation of the immune system function. Adenosine is evidenced to be generated as a result of stress response in damaged tissues and to display immunosuppressive properties (Hasko and Cronstein, 2004). A downregulation of inflammatory pathways in skeletal muscle is in contrast to the chronic low-grade pro-inflammatory state in adipose tissue (Dimitriadis et al., 2016). There is a lack of conclusive evidence, but androgens likely have direct effects on the adipose tissue resident immune cells (Huang et al., 2013). Changes in adipocyte function impair the secretion of adipokines, leading to decreased adiponectin secretion (Manneras-Holm et al., 2011), which promotes susceptibility to low grade inflammation. Despite the different immune responses in skeletal muscle and adipose tissue, it is evident that immune function is impaired in women with PCOS.

TABLE 3 | Significantly differentially expressed gene sets in skeletal muscle from women with PCOS compared with controls (GSEA, $q \leqq 0.25$).

Gene set name	Size	ES	NES	*p*	*q*
Upregulated enriched gene sets					
MYC TARGETS V1	165	0.37	1.71	0.000	0.023
SPERMATOGENESIS	46	0.45	1.71	0.000	0.043
G2M CHECKPOINT	99	0.34	1.47	0.019	0.109
HEME METABOLISM	124	0.32	1.43	0.013	0.124
MYOGENESIS	163	0.29	1.34	0.035	0.20
Downregulated enriched gene sets					
INTERFERON ALPHA RESPONSE	67	−0.57	−2.45	0.000	0.000
INTERFERON GAMMA RESPONSE	127	−0.44	−2.14	0.000	0.001
CHOLESTEROL HOMEOSTASIS	50	−0.49	−2.00	0.000	0.001
XENOBIOTIC METABOLISM	117	−0.42	−2.00	0.000	0.001
COAGULATION	66	−0.45	−1.94	0.000	0.001
EPITHELIAL MESENCHYMAL TRANSITION	130	−0.39	−1.89	0.000	0.002
APOPTOSIS	114	−0.32	−1.54	0.003	0.048
IL2 STAT5 SIGNALING	117	−0.32	−1.52	0.000	0.047
APICAL JUNCTION	111	−0.31	−1.51	0.003	0.047
COMPLEMENT	106	−0.31	−1.45	0.008	0.067
HEDGEHOG SIGNALING	16	−0.43	−1.31	0.125	0.19
INFLAMMATORY RESPONSE	86	−0.29	−1.30	0.070	0.20
HYPOXIA	134	−0.26	−1.27	0.063	0.21
TNFA SIGNALING VIA NFKB	116	−0.26	−1.24	0.083	0.25
P53 PATHWAY	136	−0.25	−1.24	0.065	0.23
ADIPOGENESIS	164	−0.24	−1.23	0.078	0.22
ALLOGRAFT REJECTION	87	−0.27	−1.22	0.130	0.24
IL6 JAK STAT3 SIGNALING	40	−0.31	−1.20	0.192	0.25
ANGIOGENESIS	22	−0.35	−1.20	0.208	0.24

The enrichment score (ES) was calculated for each gene set, the primary outcome of GSEA. NES, Enrichment score normalized for differences in gene set size.

Inflammatory and metabolic pathways in skeletal muscle are closely tied to cell signaling and differentiation, which leads to tissue specific adaptations in response to different stimuli e.g., androgens, obesity and exercise. The GSEA showed an upregulated myogenesis and the GO showed an enrichment of genes involved in negative regulation of cell proliferation, positive regulation of cell death, and negative regulation of response to stimuli, supporting skeletal muscle specific adaptations in response to PCOS. Furthermore, genes involved in MYC signaling were the most upregulated enriched gene set in the GSEA. MYC is a transcription factor that drives metabolic reprogramming in activated, primary T lymphocytes, and this may be one of the key targets responsible for metabolic reprogramming in response to PCOS conditions (Wang et al., 2011). As expected, many metabolic pathways were found to be enriched in the GO, with a negative regulation of the majority of the metabolic processes. *ALDOA*, encoding aldolase, was upregulated in women with PCOS and seems to be a key gene involved in many carbohydrate metabolic processes. Aldolase is an insulin stimulated glycolytic enzyme that catalyzes the conversion of fructose-1,6-bisphosphate during glycolysis, but it is also involved in actin remodeling, possibly modulating both metabolic and structural tissue adaptations (Hu et al., 2016).

Lastly, two genes were consistently upregulated in all three studies, *IRX3* and *CDKAL1*. Both genes have been associated with body mass index and type 2 diabetes in genome-wide association studies (Steinthorsdottir et al., 2007; Ragvin et al., 2010; Yengo et al., 2018). *IRX3* is a transcription and neuronal progenitor factor, and acts as a regulator of energy metabolism. It is shown to be controlled by a non-coding region of the fat mass and obesity-associated (FTO) gene, which is one of the strongest obesity-associated genes found in humans (Frayling et al., 2007). Many studies have investigated the role of BMI-associated variants in women with PCOS (Jones and Goodarzi, 2016). The results are controversial, but a meta-analysis showed that *FTO* has an increased effect on the BMI of women with PCOS, but it is not a PCOS susceptibility locus (Wojciechowski et al., 2012). Moreover, data for *CDKAL1* and *FTO* suggest that polymorphisms of these genes are not associated with insulin resistance or insulin secretory capacity in Asian women with PCOS (Kim et al., 2012).

The interpretation of this meta-analysis is limited by a fairly low number of women with PCOS and controls ($n <$ 40/group), the inclusion of a single ethnicity (Caucasian), and a mix of PCOS phenotypes (Rotterdam criteria). Further, our findings cannot distinguish whether the identified dysregulated

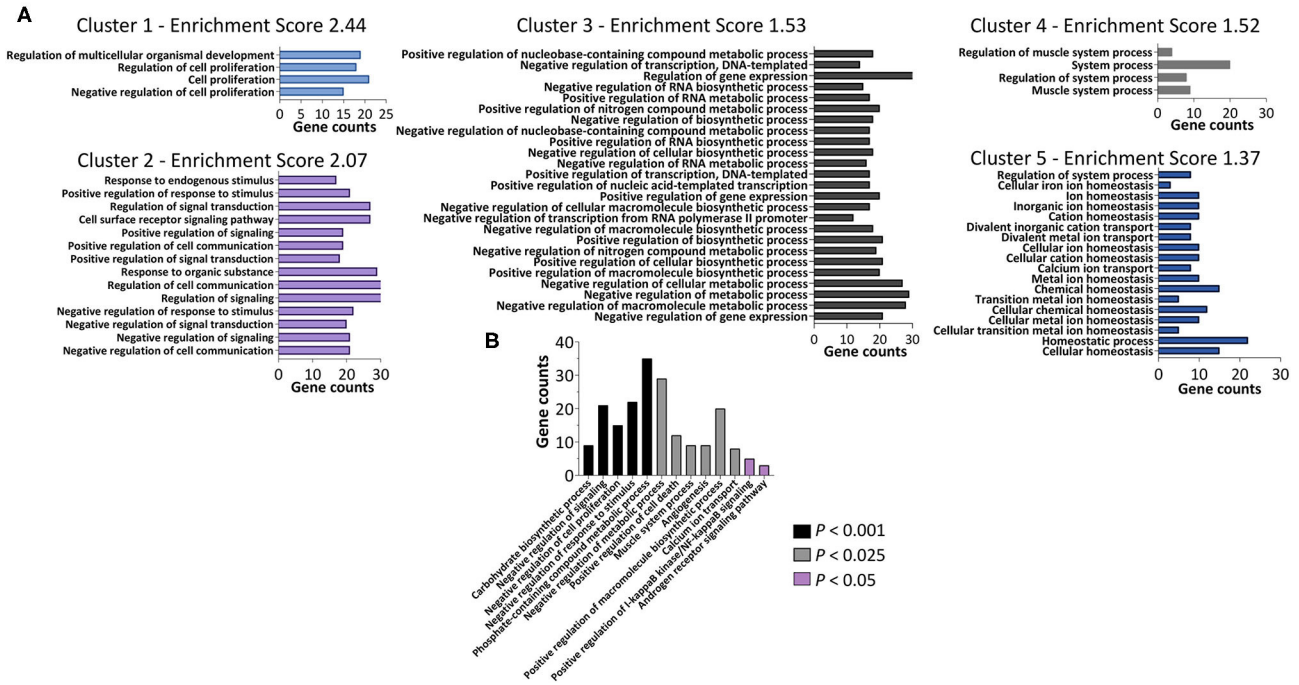

FIGURE 2 | Functional annotation clustering determined using DAVID Bioinformatics Resources with respect to the 114 differently expressed genes ($q < 0.05$) in the skeletal muscle from women with PCOS compared with controls. **(A)** The representative groups with an enrichment score of 1.3 or above are presented. The x-axis represents the number of genes, while the y-axis represents the ontology categories. **(B)** Selected GO terms of biological processes enriched by the differently expressed genes ($P < 0.05$).

pathways are responses to a stimulus or the causal effectors. Another limitation is the fact that we cannot identify the gene expression changes within specific target cells i.e., myocytes, as the muscle biopsy is a bulk of many different cell types and structures, e.g., immune cells, vessels and connective tissue.

CONCLUSION

Clinical studies and transcriptomics data clearly demonstrate that molecular dysfunction in skeletal muscle contributes to insulin resistance in women with PCOS. Intracellular insulin-signaling pathways, mitochondrial function and fat oxidation, with a possible contribution of reduced adiponectin levels, have all been in focus for the mechanism of insulin resistance in PCOS (Stepto et al., 2019). Here, we identify 99 genes not previously shown to be altered in PCOS muscle, two of them consistently upregulated in all three studies. We show that immunometabolism can be added to the list of dysfunctional pathways and present genes with large effect size that warrant further investigation. However, gene expression does not always translate to alterations in biological function and data on total and phosphorylated protein levels in skeletal muscle is limited. Therefore, functional studies and proteomics analysis is needed to validate and advance our understanding of insulin resistance in skeletal muscle in these women.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

MM, ES-V, and AB designed the study and contributed to the interpretation of data. MM and AB performed data analyses and statistical analyses, and wrote the manuscript. All authors critically revised and approved the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.573505/full#supplementary-material

Supplementary Figure 1 | PRISMA flow chart.

Supplementary Table 1 | PRISMA check list.

Supplementary Table 2 | Individual analyses of transcripts in dataset 1 (Skov et al., 2007, 2008) and 2 (Nilsson et al., 2018) ($P < 0.01$).

Supplementary Table 3 | Overrepresentation analysis using gene ontology (GO) on the 114 genes differentially expressed in skeletal muscle from women with PCOS compared with controls ($q < 0.05$).

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TMEPAI/PMEPA1 Is a Positive Regulator of Skeletal Muscle Mass

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Inhibition of myostatin- and activin-mediated SMAD2/3 signaling using ligand traps, such as soluble receptors, ligand-targeting propeptides and antibodies, or follistatin can increase skeletal muscle mass in healthy mice and ameliorate wasting in models of cancer cachexia and muscular dystrophy. However, clinical translation of these extracellular approaches targeting myostatin and activin has been hindered by the challenges of achieving efficacy without potential effects in other tissues. Toward the goal of developing tissue-specific myostatin/activin interventions, we explored the ability of transmembrane prostate androgen-induced (TMEPAI), an inhibitor of transforming growth factor-β (TGF-β1)-mediated SMAD2/3 signaling, to promote growth, and counter atrophy, in skeletal muscle. In this study, we show that TMEPAI can block activin A, activin B, myostatin and GDF-11 activity *in vitro*. To determine the physiological significance of TMEPAI, we employed Adeno-associated viral vector (AAV) delivery of a TMEPAI expression cassette to the muscles of healthy mice, which increased mass by as much as 30%, due to hypertrophy of muscle fibers. To demonstrate that TMEPAI mediates its effects via inhibition of the SMAD2/3 pathway, tibialis anterior (TA) muscles of mice were co-injected with AAV vectors expressing activin A and TMEPAI. In this setting, TMEPAI blocked skeletal muscle wasting driven by activin-induced phosphorylation of SMAD3. In a model of cancer cachexia associated with elevated circulating activin A, delivery of AAV:TMEPAI into TA muscles of mice bearing C26 colon tumors ameliorated the muscle atrophy normally associated with cancer progression. Collectively, the findings indicate that muscle-directed TMEPAI gene delivery can inactivate the activin/myostatin-SMAD3 pathway to positively regulate muscle mass in healthy settings and models of disease.

Keywords: Gdf11, myostatin, activin, cachexia, muscle, PMEPA1, TMEPAI

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INTRODUCTION

Myostatin, activin A, activin B, and GDF11 comprise a subgroup within the transforming growth factor-β (TGF-β) family of proteins that is of great interest in the field of skeletal muscle biology (Chen et al., 2016a). These secreted ligands negatively regulate the morphogenesis and growth of skeletal muscle by binding to activin type II receptors (ActRIIA or ActRIIB), and then recruiting and activating type I receptors (ALK4 or ALK5; Chen et al., 2016a). Activated type I receptors initiate signaling via SMAD2/3 transcription factors, which limit muscle growth via effects on both protein synthesis and protein degradation pathways. Expression of myostatin or activin A is sufficient to inhibit anabolic signaling associated with Akt/mTOR activity (Amirouche et al., 2009; Chen et al., 2014). Concurrently, SMAD2/3 activation regulates the expression of the E3 ubiquitin ligases *MuRF-1* and *Atrogin-1* (Sartori et al., 2009; Lokireddy et al., 2011; Goodman et al., 2013), which mediate ubiquitination and proteasomal degradation of myofibrillar proteins, such as myosin (Clarke et al., 2007; Fielitz et al., 2007). Ultimately, these ActRIIA/B ligands regulate genes associated with muscle protein turnover, metabolism, and sarcomere function (Chen et al., 2015, 2017) that are transcriptionally indicative of muscle wasting associated with cancer cachexia (Baker et al., 2010; Johnston et al., 2015; Rovira Gonzalez et al., 2019).

While physiological activity of these TGF-β ligands contributes to the homeostatic maintenance of muscle mass (Chen et al., 2017), elevated circulating levels are highly catabolic, inducing significant muscle wasting in murine models. For example, elevating systemic levels of myostatin, activin A or GDF11, by injecting Chinese hamster ovary (CHO) cells expressing these proteins into the quadriceps of athymic nude mice, results in a global decline in skeletal muscle mass of >20% within 12 days (Zimmers et al., 2002, 2017; Walton et al., 2019). Similar results can be observed when adeno-associated viral vectors (AAV vectors) delivered to the muscles of mice are used to elevate circulating activin A levels (Chen et al., 2014). These findings are significant because these SMAD2/3-activating ligands, particularly activin A and activin B, are elevated in many chronic conditions where muscle wasting is observed, including cancer, sepsis, lung disease, and heart disease (Michel et al., 2003; Yndestad et al., 2004, 2009; Loumaye et al., 2015, 2017). Tellingly, high activin A/B levels are predictive of adverse outcome in patients with acute respiratory failure (de Kretser et al., 2013). Thus, concerted efforts have been directed toward developing therapies that can block SMAD2/3 activity and preserve muscle mass in chronic disease.

To date, soluble activin type II receptors (sActRIIA, sActRIIB), ligand-targeting antibodies, and follistatin have proven the most efficacious therapeutic reagents in mice (Zhou et al., 2010; Winbanks et al., 2012; Latres et al., 2017), and humans (Attie et al., 2013; Campbell et al., 2017). These molecules potently antagonize signaling by activin-related ligands and reverse muscle wasting in cancer cachexia models (Li et al., 2007; Benny Klimek et al., 2010; Zhou et al., 2010). However, a recent phase II clinical trial using sActRIIB in patients with muscular dystrophy was halted when some participants experienced bleeding from mucosal surfaces (Campbell et al., 2017). This side-effect likely arises from sActRIIB inhibiting the anti-angiogenic properties of multiple TGF-β ligands (David et al., 2007) and highlights a problem associated with the use of systemically-administered pleiotropic antagonists to treat muscle wasting conditions.

An alternative approach is to identify cytoplasmic/membrane-associated skeletal muscle proteins that may inhibit SMAD2/3 activation. To this end, we recently identified *Pmepa1* as a transcriptional target of activin A signaling in skeletal muscle (Chen et al., 2014). *Pmepa1* encodes the transmembrane prostate androgen-induced (TMEPAI) protein, which is expressed in many tissues. Elevated expression of TMEPAI has been identified in tumor biopsies and cell lines derived from patients with prostate, colorectal, breast, ovarian, lung and kidney cancers (Xu et al., 2000; Rae et al., 2001; Brunschwig et al., 2003; Giannini et al., 2003). Evidence indicates that TMEPAI expression in these tumors acts to counteract cell proliferation (Singha et al., 2014; Fournier et al., 2015; Li et al., 2015; Du et al., 2018; Abdelaziz et al., 2019), but this activity is highly dependent on tumor origin (Rae et al., 2001; Giannini et al., 2003; Vo Nguyen et al., 2014; Singha et al., 2019).

TMEPAI exerts its activity, in part, by inhibiting the SMAD2/3 signaling pathway, at the level of the plasma membrane (Watanabe et al., 2010). To date, this action of TMEPAI has only been demonstrated in relation to TGF-β1 signaling (Watanabe et al., 2010; Nakano et al., 2014). In this context, TMEPAI competes with the adaptor protein Smad anchor for receptor activation (SARA) for binding to SMAD2. This competition prevents SARA from recruiting SMAD2 to the TGF-β type I receptor (TβR1/ALK5) and, thereby, reduces the TGF-β1 transcriptional response (Watanabe et al., 2010). TMEPAI exerts this activity via a short SMAD-inhibitory domain (SIM), which lies within its C-terminal cytoplasmic region. Targeted disruption of the SIM domain inhibits the ability of TMEPAI to block TGF-β1-mediated SMAD2/3 signaling. The SIM domain in TMEPAI is flanked by two "PY" motifs (PPXY), which are dispensable for inhibition of TGF-β1 signaling but required for regulation of Akt signaling pathways (Watanabe et al., 2010). Although there is some evidence to support that TMEPAI is capable of also blocking activin A-mediated biological effects (Watanabe et al., 2010), the extent to which TMEPAI can regulate signaling mechanisms in skeletal muscle has not been determined.

In this study we aimed to test the hypothesis that TMEPAI can attenuate myostatin/activin signaling in skeletal muscle fibers to promote hypertrophy and attenuate muscle wasting. We generated an AAV vector expressing TMEPAI and tested its capacity to increase muscle mass following intramuscular injection in healthy mice, and to preserve muscle mass in settings of muscle wasting associated with increased activin A expression. Our studies provide the first evidence that muscle-directed expression of TMEPAI can attenuate the atrophic actions of activin/myostatin activity with beneficial consequences for the maintenance of skeletal muscle mass.

MATERIALS AND METHODS

Generation of TMEPAI Constructs

A pCDNA 3.1 mammalian vector comprising the full length human TMEPAI sequence (reference sequence NM_020182.4) was obtained from GenScript (New Jersey, United States). A C-terminal polyhistidine-tag (8x histidine) was first introduced by PCR, to facilitate downstream purification of TMEPAI. The polyhistidine-tag was incorporated by PCR using an N-terminal TMEPAI primer (sense sequence 5′-CTAGAAGCTT ATGCACCGCTTGATGGGGGTCAACAGCAC-3′) with a C-terminal poly-histidine primer (anti-sense sequence 5′-CTA GGCGGCCGCCTAATGATGGTGGTGATGGTGATGATGGAG AGGGTGTCCTTTCTGTTTATCCTTCTCTTTGCTCCAGATG GC-3′). The primers incorporated restriction sites for *Hind*III and *Not*I, respectively, to enable insertion of the resulting TMEPAI+HIS PCR product into these sites of the pCDNA 3.1 vector. Point mutations in the PY-domains of TMEPAI were generated using the Quik-Change Lightning mutagenesis kit (Agilent Technologies), according to the manufacturer's guidelines. Primers used for this mutagenesis were as follows: TMEPAI-SIM (sense 5′-GGTGCGCGCACCCGCTGCAAGAAC CATCTTCGAC-3′ and anti-sense 5′-GTCGAAGATGGTTC TTGCAGCGGGTGCGCGCACC-3′), TMEPAI-PY1 (sense 5′-GAGCCCCCACCCGCTCAGGGCCCCTGCAC-3′ and anti-sense 5′-GTGCAGGGGCCCTGAGCGGGTGGGGGCTC-3′), and TMEPAI-PY2 (sense 5′-GCCGCCGCCCACCGCTAGCG AGGTCATCG-3′ and anti-sense 5′-CGATGACCTCGCTAGCG GTGGGCGGCGGC-3′). Introduced mutations were confirmed using DNA sequencing.

Expression of TMEPAI Variants in HEK293T Cells

Production of TMEPAI protein variants was verified by transient transfection in human embryonic kidney (HEK293T) cells cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), 100 mM sodium pyruvate and 1% penicillin/streptomycin (all reagents from Thermo Fisher Scientific, Massachusetts, United States). For transfection, HEK293T cells were plated at 8×10^5 cells/well in 6-well plates and incubated overnight at 37°C. The following day, 5 μg of TMEPAI construct DNA was combined with Lipofectamine 2000 (Thermo Fisher Scientific) in OPTI-MEM media and added to the cells as outlined by the manufacturer. Following a 24-h incubation, the cells were lysed for western blot analysis (as described below).

Assessment of TMEPAI Bioactivity *in vitro*

The ability of TMEPAI to block SMAD2/3-mediated ligand signaling was assessed in a HEK293T luciferase bioassay. In brief, HEK293T cells were first seeded into a 48-well plate at a density of 7.5×10^4 cells/well in DMEM containing 10% FCS (without antibiotics). At 24 h post-plating, cells were transfected with the SMAD2/3-responsive A3-Luciferase reporter construct and FAST2 transcription factor (Liu et al., 1999) as well as increasing amounts of either wild-type or mutant TMEPAI constructs (0–9 ng/well), using Lipofectamine 2000 (Thermo Fisher Scientific). At 24 h post-transfection, cells were treated with 200 pM of various SMAD2/3 activating ligands (myostatin, activin A, activin B, and GDF11, all sourced from R&D Systems, Minnesota, United States, or TGF-β1 from PeproTech, United States). The following day, cells were harvested in solubilisation buffer (1% Triton X-100, 25 mM glycylglycine, pH 7.8, 15 mM MgSO4, 4 mM EGTA, and 1 mM dithiothreitol), and the SMAD2/3-induced luciferase activity was determined. Luciferase activity was determined as a % control of the ligand stimulated cells. Ligand treatments typically resulted in a 10- to 30-fold increase in basal luciferase activity. Total Area Under the Curve was compared for mutant TMEPAI forms (SIM and PY1 + PY2) relative to wild type TMEPAI luciferase activities. Values are total area ± standard error. Significance was determined using two-way ANOVAs with Tukey's *Post Hoc* analysis.

To support this analysis, the inhibition of ligand-mediated phosphorylation of SMAD2 and SMAD3 by the TMEPAI variants was determined by western blot. In brief, HEK293T cells were plated at 4×10^5 cells/well in 12-well plates in DMEM media with 10% FCS (without antibiotics). The following day, the cells were transfected with TMEPAI constructs (0 or 1 μg DNA/well) using Lipofectamine 2000. After a 24-h incubation, cell culture media was replaced with low serum media (DMEM, 0.2% FCS and 50 mM HEPES) and incubated for 4 h at 37°C to suppress basal activation of pSMAD2/3. Cells were then treated with 200 pM activin A or TGF-β1 (diluted in low serum media) and incubated for 45 min. The treated HEK293T cells were subsequently processed for western blotting as described below.

Western Blotting

HEK293T cells were lysed in ice cold RIPA buffer (10 mM Tris-Cl, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS and 140 mM NaCl, pH 8.0) containing Complete protease inhibitors and PhosStop phosphatase inhibitors (both Roche Applied Sciences). Skeletal muscles were homogenized in ice cold lysis buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, 1% SDS, 1% Triton X-100, and phosphatase (P5726, Sigma Aldrich, Missouri, United States) and protease (P8340, Sigma) inhibitors. Following lysis, samples were rotated for 20 min at 4°C, incubated at 70°C for 10 min with frequent mixing and centrifuged at 18,000 *g* for 20 min at 4°C. The protein concentration of resulting supernatants was determined using a BCA protein assay (Thermo Fisher Scientific), samples were reduced with a final concentration of 2.5% β-mercaptoethanol and then denatured for 5 min at 95°C. Protein fractions were resolved by SDS-PAGE using pre-cast 10% Tris-Glycine (Bio-Rad, California, United States) or 4–12% Bis-Tris gels (Thermo Fisher Scientific), blotted onto nitrocellulose or PVDF membranes (Bio-Rad), blocked for a minimum of 1 h in either 5% skim milk powder or 1% BSA in TBS-T (50 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 8.0) and incubated overnight at 4°C in primary antibody solutions. All primary antibodies were used at a dilution of 1:1000 unless otherwise stated. Antibodies

against pSmad2$^{Ser465/467}$ (#3101), total SMAD2/3 (#8685), and GAPDH-HRP (#3683) were purchased from Cell Signaling Technologies (Massachusetts, United States). Antibodies targeting pSmad3$^{Ser423/425}$ (#ab52903) were purchased from Abcam (Cambridge, United Kingdom). Antibodies targeting Filamin C (1:2,000) were purchased from Sigma Aldrich. TMEPAI was detected using an anti-PMEPA1/TMEPAI antibody (#85829 Santa Cruz Biotechnology, United States) at 1:1000. Bound primary antibodies were detected by incubation with HRP-conjugated secondary rabbit (#7074, Cell Signaling Technologies), mouse (#NXA931V, Amersham, United Kingdom), or goat antibodies (#P0448, Agilent/Dako, United States) in 5% skim milk powder or 1% TBS-T for 1 h. Chemiluminescence was detected using ECL western blotting detection reagents (GE Healthcare, Buckinghamshire, United Kingdom) and Immobilon Forte western HRP substrate (Merck, New Jersey, United States).

Production of AAV Vectors

cDNA constructs encoding TMEPAI or activin A (ActA) were cloned into AAV expression plasmids consisting of a cytomegalovirus (CMV) or the tetracycline response element (TRE) promoter and SV40 poly-A region flanked by AAV2 terminal repeats. Viral vector production was performed as described previously (Blankinship et al., 2004). Briefly, HEK-293 cells were plated at a density of 7.2–8.5 $\times$ 10^6 cells on a 15 cm culture dish and incubated for 8–16 h. The cells were then transfected with 22.5 μg of a vector genome-containing plasmid and 45 μg of the packaging/helper plasmid pDGM6 using calcium phosphate precipitation. At 72 h post transfection, the media and cells were collected and lysed via three freeze-thaw cycles before 0.22 μm clarification (Merck). Purification of viral vector particles from crude lysates was performed using affinity chromatography via heparin affinity column (HiTrap, Amersham) and ultracentrifugation overnight prior to re-suspension in sterile physiological Ringer's solution. The purified vector preparations were titered with a customized sequence-specific quantitative PCR-based reaction (Thermo Fisher Scientific).

Animal Experiments

All experiments were conducted in accordance with the relevant code of practice for the care and use of animals for scientific purposes (National Health and Medical Council of Australia). All experimental protocols were approved by the Alfred Medical Research and Education Precinct Animal Ethics Committee (AMREP AEC) and University of Melbourne Animal Ethics committee. Cohorts of 8-week-old C57Bl/6J mice were obtained from the breeding colony maintained by the AMREP Animal Services facility (Melbourne, Australia), the founders of which were sourced from the Jackson Laboratory. Cohorts of 7-week-old BALB/c mice were sourced from the Walter and Eliza Hall Institute Bioservices Kew Division. Mice were fed standard chow diets unless otherwise stated, with access to drinking water *ad libitum* while housed under a 12:12-h light dark cycle. All surgical procedures were performed on mice placed under general anesthesia via inhalation of isoflurane in medical oxygen supported by post-operative analgesia. AAV doses administered to mice were determined from dose optimization experiments. AAV:TMEPAI was administered to mice at a dose of 1 $\times$ 10^9 vg. For studies involving the over-expression of activin A, a tetracycline-inducible gene expression construct (AAV:TetOn) and a TetOn-responsive ActA (AAV:TRE-ActA) construct were administered at doses of 1 $\times$ 10^{10} vg and 1 $\times$ 10^9 vg, respectively. Control treatments consisted of delivery of a viral vector lacking a functional gene (Con) into the contralateral limb at equivalent vector genome doses. Vectors were diluted in 30 μl of Hank's buffered saline solution and injected into the anterior compartments of the lower hind limb of anesthetized mice. Following AAV:TetOn and AAV:TRE-ActA administration, mice were provided standard chow containing 600 mg/kg doxycycline (Specialty Feeds), to facilitate sustained gene expression. C57Bl/6J mice administered AAV:TRE-ActA, AAV:TetOn and AAV:TMEPAI were analyzed 4 weeks after treatment. Implantation of C26 solid tumor tissue into the flank of BALB/c mice was performed, as previously described (Aulino et al., 2010; Winbanks et al., 2016). Tumor-bearing mice were treated with AAV:TMEPAI and AAV:Control at the time of tumor implantation. The experimental endpoint was determined by an ethical criterion of ∼25% loss of initial body mass. At the experimental endpoint, mice were humanely euthanized via overdose of sodium pentobarbitone (100 mg/kg) or cervical dislocation. Skeletal muscles, tissues and organs were excised rapidly and weighed before subsequent processing.

Skeletal Muscle Histology

Portions of harvested muscles were placed in OCT cryoprotectant (ProSciTech, Queensland, Australia) and frozen in liquid nitrogen-cooled isopentane. Frozen samples were cryosectioned at 10 μm thickness for hematoxylin and eosin staining as previously described (Hagg et al., 2016). Sections were mounted using DePeX mounting medium (VWR, Leicestershire, England) and imaged at room temperature using a U-TV1X-2 camera mounted to an IX71 microscope and a PlanC 10X/0.25 objective lens (Olympus). Images of sections were obtained using acquisition software (DP2-BSW, Olympus). Analysis of myofibre diameter was performed on muscle samples cryosectioned at 8 μm thickness. Sections were incubated in FITC blocking buffer (Thermo) for 30 min and then in Alexa Fluor 555-conjugated Wheat Germ Agglutinin (WGA; diluted in PBS) for 2 h. Sections were stained with DAPI, mounted in Mowiol 4-88 and imaged using a fluorescence microscope (Axio Imager M2, Zeiss). The minimum Feret's diameter of muscle fibers was determined by measuring at least 400 fibers per mouse muscle, using ImageJ software (United States National Institutes of Health, Bethesda, MD, United States).

Quantitative PCR Analysis of Target Genes

Total RNA was isolated from skeletal muscles using TRIzol (Thermo Fisher Scientific) according to manufacturer's instructions. First, 1 μg of RNA was reverse transcribed

using the High Capacity RNA-to-cDNA kit (Thermo Fisher Scientific). Gene expression was analyzed by qRT-PCR using SYBR primers (Sigma and Thermo Fisher Scientific) and Real-Time PCR detection (CFX384, Bio-Rad). Target gene expression was normalized to *Hprt*. Data were analyzed using the $\Delta\Delta$CT method of analysis and are presented as fold change of the control sample (expressed as 1). Oligonucleotide primer sequences used are listed in **Table 1**.

Experimental Design and Statistical Analysis

In vitro studies were replicated three times. *In vivo* studies were performed once. Exclusion criteria for animals were applied in the case of death, cannibalism or tumor ulceration. Exclusion criteria for samples were applied in the case of histological artifacts (freeze- and sectioning-damage) and RNA or protein degradation. One- and two-way ANOVAs were used to assess statistical differences between more than two conditions, with the Tukey's *post-hoc* test used for comparisons between the specific group means (GraphPad Prism v.6, La Jolla, CA, United States). Data are presented as the means ± SEM. Comparisons between two conditions utilized the Student's *t*-test. Statistically significant changes are denoted by asterisks, level of significance is outlined in individual figure legends. Significance denoted by $^{****}p < 0.0001$, $^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$.

RESULTS

TMEPAI Inhibits the *in vitro* Activity of Multiple SMAD2/3-Activating Ligands

In previous studies conducted by our laboratories, transcriptional profiling indicated that skeletal muscle expression of TMEPAI mRNA (*Pmepa1*) increased following exposure to activin A (Chen et al., 2014) and decreased when activin and/or myostatin signaling pathways were inhibited (Chen et al., 2017). In this study, qPCR analysis of mouse tibialis anterior (TA) muscles injected with AAV:ActA confirmed that TMEPAI transcription increased markedly (6-fold) in response to activin A expression (**Supplementary Figure 1A**). This evidence supports the hypothesis that activin A, like TGF-β1, can transcriptionally activate TMEPAI, likely via their shared SMAD2/3 intracellular pathway.

TMEPAI provides negative feedback for TGF-β1-mediated SMAD2/3 signaling (Watanabe et al., 2010; Nakano et al., 2014), however, its ability to restrict the activity of related SMAD2/3 activators has not been explored. To test this hypothesis, we transfected cells with constructs expressing wild-type TMEPAI and versions containing mutations in the SIM and PY1/PY2 domains implicated in protein-protein interactions, (**Figures 1A,B**) and examined SMAD2/3 signaling activity in response to ligand exposure (**Figures 1C–G**). We found that TMEPAI also suppresses SMAD2/3-induced luciferase expression triggered by myostatin, activin A, activin B, and GDF-11, in addition to TGF-β1 (**Figures 1C–G**). Transient transfection of cells with constructs expressing TMEPAI (**Figure 1B**) exerted inhibitory activity via the tryptophan-rich SIM domain (**Figure 1A**), as mutation of this region (PPNR to PPAA) abolished the ability of TMEPAI to block luciferase activity induced by all ligands tested (**Figures 1C–G** and **Table 2**). In contrast, flanking PY1/PY2 motifs within TMEPAI, thought to mediate interactions with the ubiquitin ligase NEDD4 (Watanabe et al., 2010), were not required for TMEPAI inhibitory activity against these ligands (**Figures 1C–G**). Mechanistically, TMEPAI blocks both endogenous SMAD2 and SMAD3 pathways, as shown by suppression of activin A-induced SMAD2/3 phosphorylation in HEK293T cells (**Figures 1H,I**). TMEPAI also blocked TGFβ1-induced SMAD3 phosphorylation in HEK293T cells, however, the cells were unresponsive to TGFβ1-induced SMAD2 activation (**Figures 1H–K**). These findings demonstrate that TMEPAI can inhibit SMAD2/3 activation by myostatin, activin A, activin B and GDF-11 via protein interactions dependent on the TMEPAI SIM domain.

TABLE 1 | Oligonucleotide sequences used to perform qRT-PCR.

Gene	Fwd sequence	Rev sequence
mTmepai	GAAGGCAAAAGAGAAAATGC	GAAATTAAGCATTCACGCAC
mIgfn1	CCTCATTGTCACAGAATACG	ATTTGCCATCCATCTCATAG
mCyr61	AGAGGCTTCCTGTCTTTG	GTTGTCATTGGTAACTCGTG
mMurf1	ACCTGCTGGTGGAAAACATC	CTTCGTGTTCCTTGCACATC
mAtrogin1	GCAAACACTGCCACATTCTCTC	CTTGAGGGGAAAGTGAGACG
mHprt	GTTTGTTGTTGGATATGCCCTTG	GGCAACATCAACAGGACTCC

TMEPAI Promotes Skeletal Muscle Hypertrophy and Blocks Activin A-Mediated Muscle Wasting *in vivo*

Basal SMAD2/3 activation by endogenous myostatin and activins restricts muscle growth (Chen et al., 2017). Additionally, overexpression of myostatin, activin A, and activin B, but not TGF-β1, induces muscle atrophy (Chen et al., 2014). Therefore, we sought to test the hypothesis that increased expression of TMEPAI in muscle would counteract the ligands that negatively regulate muscle mass via SMAD2/3 activation. Here, we found that delivery of AAV:TMEPAI to the TA hindlimb muscles of mice promoted muscle hypertrophy, as demonstrated by a 12% increase in TA mass (**Figures 2A,B**) and 16% increase in muscle fiber size (**Figures 2C,D**). We have shown that elevated circulating levels of activin A, similar to those observed in patients with cancer cachexia (Loumaye et al., 2015) and in mouse models of cancer cachexia, can induce profound muscle wasting (Chen et al., 2014). Therefore, we examined whether TMEPAI could protect skeletal muscle against activin A-induced atrophy. Examination of C57Bl/6J male mice 4 weeks after intramuscular injection of an AAV vector expressing activin A (AAV:ActA) identified a 30% reduction in TA muscle mass compared with contralateral muscles (**Figures 2A,B**). In contrast, co-delivery of AAV:TMEPAI with AAV:ActA attenuated activin A-induced loss of muscle mass (**Figures 2A,B**). Histological analysis indicated that TMEPAI protected muscle fibers from activin A-mediated atrophy (**Figures 2C,D**). In terms of mechanism,

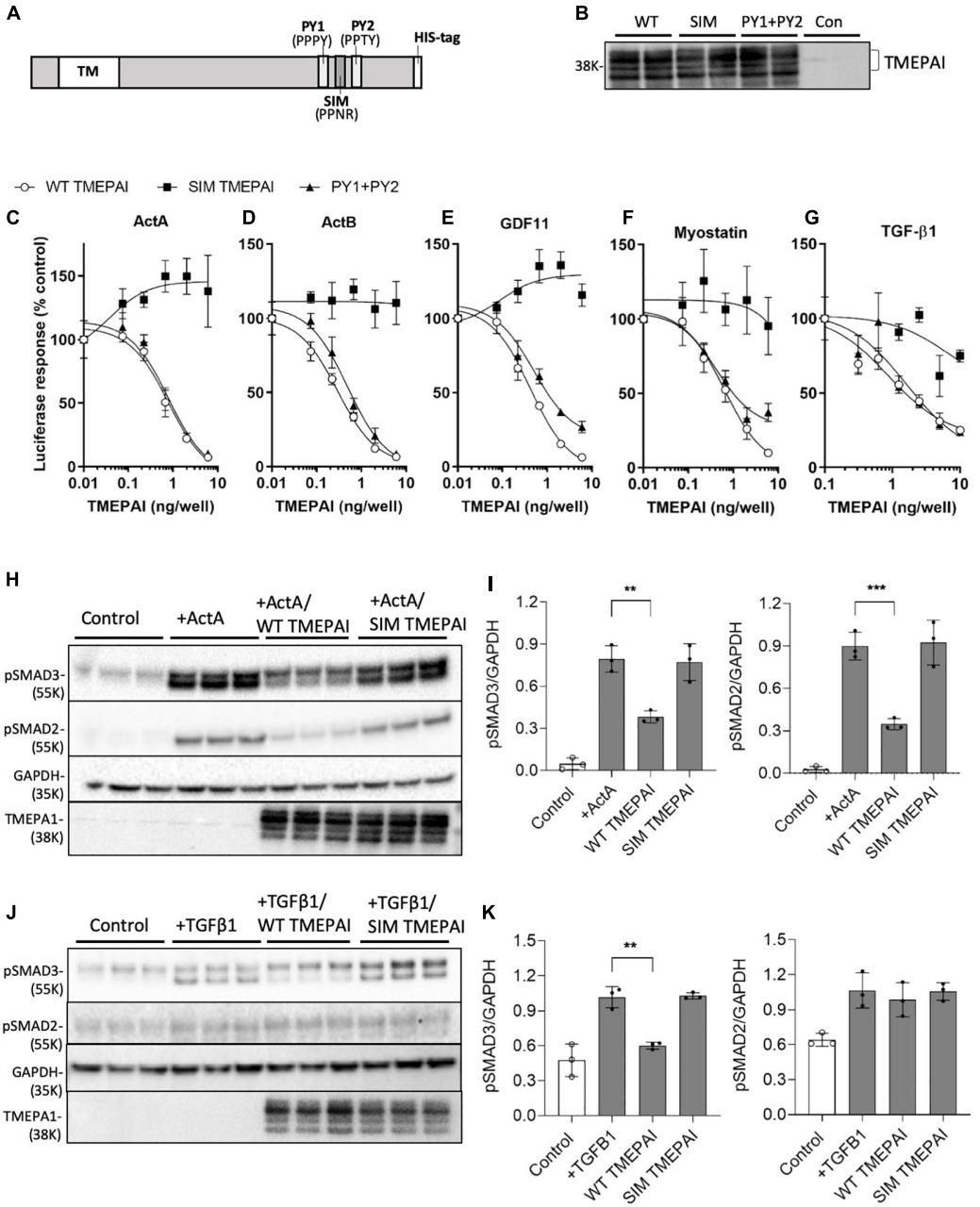

FIGURE 1 | TMEPAI inhibits the *in vitro* activity of multiple SMAD2/3 ligands via its SIM domain. **(A)** Schematic model of TMEPAI noting transmembrane (TM), SMAD2/3 inhibitory domain, pAkt PY motifs (PY) and placement of an inserted polyhistidine (HIS) tag. **(B)** Expression of TMEPAI variants in transfected HEK293T mammalian cells. Reduced cell lysates were probed with an anti-TMEPAI antibody. Multiple TMEPAI protein species are likely owing to differential posttranslational modifications. **(C–G)** The ability of TMEPAI to block ligands that signal through SMAD2/3 was determined using a SMAD2/3 responsive luciferase reporter assay in HEK293T cells. Cells were transfected with plasmids encoding for the luciferase reporter and increasing doses of TMEPAI, then stimulated with the ligands **(C)** activin A, **(D)** activin B, **(E)** GDF-11, **(F)** Myostatin, and **(G)** TGF-β1. Luciferase activity was determined as a measure of SMAD2/3 induction ($n = 3$ samples/treatment, representative result of $n = 3$ replicates). **(H, I)** Western blot analysis of HEK293T lysates revealing that inactivation of the SIM domain in TMEPAI inhibits the ability of TMEPAI to block activin A-induced endogenous SMAD2 and SMAD3 phosphorylation. **(J, K)** TMEPAI also blocks TGFβ1-induced endogenous SMAD3 phosphorylation, but not SMAD2 in HEK293T cells. For western blots, $n = 3$ samples/treatment condition, $^{***}p < 0.001$ and $^{**}p < 0.01$.

we analyzed SMAD3 phosphorylation in the TA muscles of mice expressing activin A in the presence, or absence, of TMEPAI. Western blot analysis of muscle lysates indicated that TMEPAI suppressed activin A-mediated phosphorylation of pSMAD3 (**Figures 2E–G**). Additionally, TMEPAI potently inhibited activin-induced upregulation of SMAD2/3-target genes *Cyr61* and *Igfn1* (**Figures 2H,I**). Together, these *in vivo* studies demonstrate that TMEPAI inhibits the SMAD3-mediated

TABLE 2 | Luciferase assay data – Area Under the Curve analyses.

	WT TMEPAI	SIM TMEPAI	PY1 + PY2
Activin A	192.1 ± 7.9	368 ± 12.8****	202 ± 9^{ns}
Activin B	147.2 ± 6.1	307.8 ± 8.9****	177 ± 6^{ns}
GDF11	169.1 ± 4.8	328.9 ± 6.1****	205 ± 4^{ns}
Myostatin	184.4 ± 5.1	304.3 ± 14.3***	199 ± 9^{ns}
TGF-β1	117.7 ± 18.2	178.9 ± 9.1***	126 ± 6^{ns}

*Total Area Under the Curve (arbitrary values) was compared for mutant TMEPAI forms (SIM and PY1 + PY2) relative to wild type TMEPAI. Values are total area ± standard error. Significance was determined using two-way ANOVAs with Tukey's Post Hoc analysis (n = 3 samples). ****p < 0.0001, ***p < 0.001, and ns = not significant.*

transcriptional response initiated by exogenous, activin A, thereby, protecting muscle mass.

TMEPAI Preserves Muscle Mass in a Mouse Model of Cancer Cachexia

As myostatin and activins have been implicated in the pathogenesis of cancer cachexia, we next sought to determine if TMEPAI could prevent loss of muscle mass in a mouse model of cancer cachexia, induced by the subcutaneous growth of an implanted colon-26 (C26) carcinoma in male BALB/c mice (Aulino et al., 2010; Zhou et al., 2010; Winbanks et al., 2016). In the absence of tumor growth, intramuscular injection of AAV:TMEPAI promoted a significant increase in TA mass (21%) and fiber size (20%) in BALB/c mice (**Figures 3A,B**), which was similar to the changes observed in C57Bl/6J mice (**Figures 2A,B**). BALB/c mice bearing C26 tumors displayed a 32% reduction in TA mass, however, intramuscular administration of TMEPAI significantly attenuated muscle loss (**Figures 3A,B**). TMEPAI-expressing TA muscles in C26 tumor-bearing mice were on average only 12% reduced in mass when compared with the muscles of tumor-free mice (**Figures 3A,B**). Histological analysis supported these findings, demonstrating a greater average fiber diameter in muscles from C26 tumor-bearing mice administered AAV:TMEPAI, compared to contralateral limb muscles administered AAV:Control (**Figures 3C,D**). This average myofibre size was larger than that observed in the TA muscles of tumor-free control mice (**Figures 3C,D**). Phosphorylation of SMAD3 was significantly reduced in the presence of TMEPAI in the muscles of C26 tumor-bearing mice (**Figures 3E–G**), and TMEPAI expression also attenuated transcriptional activation of SMAD2/3-target genes in C26 tumor-bearing mice, including *Cyr61,* and *Igfn1* (**Figures 3H,I**). Additionally, TMEPAI expression suppressed transcription of the muscle atrophy genes *MuRF-1* and *Atrogin-1* (**Figures 3J,K**).

DISCUSSION

Due to their potent actions on muscle and other tissues, ligands that signal via activin type II receptors are amongst the most highly regulated members of the TGF-β superfamily. Myostatin is secreted in a latent complex with its prodomain and requires activation by tolloid metalloproteases (Wolfman et al., 2003). However, activation alone does not guarantee receptor binding, as numerous extracellular binding proteins, including follistatin, follistatin-like 3, GASP-1 and GASP-2, inhibit myostatin activity (Chen et al., 2016a). While activin A and activin B are secreted in active forms, they too are neutralized by follistatin and follistatin-related proteins (Nakamura et al., 1990; Tsuchida et al., 2000) and receptor access can be blocked by inhibin A and inhibin B (Lewis et al., 2000). Many of these antagonists and soluble forms of the activin type II receptor have been shown to increase muscle mass in healthy mice and mice modeling muscle wasting conditions (Li et al., 2007; Zhou et al., 2010; Winbanks et al., 2012; Parente et al., 2020). However, the potential side effects of molecules that target ligands with broad expression and cell tropism such as activin A/B (Walton et al., 2012; Campbell et al., 2017) may limit their therapeutic utility.

An alternative approach to inhibit myostatin/activin activity is to limit SMAD2/3 phosphorylation downstream of activin type I receptors in cells of interest. We previously demonstrated the potential of this approach by using AAV vectors to increase expression of SMAD7 in skeletal muscle fibers (Winbanks et al., 2016). SMAD7 prevents SMAD2/3 phosphorylation, promotes ActRIIB degradation (Hayashi et al., 1997), and was observed to increase muscle mass in healthy mice and attenuate wasting in models of cachexia (Winbanks et al., 2016). Other SMAD2/3 inhibitory proteins, including Smurf, c-Ski, SnoN, TGIF, and TMEPAI, have primarily been studied in the context of TGF-β1 signaling (Akiyoshi et al., 1999; Stroschein et al., 1999; Wotton et al., 1999; Zhu et al., 1999). However, the recent observation that activin induces TMEPAI expression in skeletal muscle (Chen et al., 2014) suggested that this membrane-associated protein may be involved in an intracellular negative feedback system that limits the activation of SMAD2/3 and, thereby, helps maintain muscle homeostasis.

TMEPAI is a membrane- or lysosome-associated protein that regulates not only TGF-β1 signaling but also androgen receptor (AR) and phosphatase and tensin homologue deleted on chromosome 10 (PTEN) activity (Watanabe et al., 2010; Singha et al., 2014). Regulation of these pathways help to explain a link between TMEPAI and tumourigenicity, where it has been shown TMEPAI can either limit cancer progression and metastases (Singha et al., 2014; Fournier et al., 2015; Li et al., 2015; Du et al., 2018; Abdelaziz et al., 2019) or promote cancer development (Rae et al., 2001; Giannini et al., 2003; Vo Nguyen et al., 2014; Singha et al., 2019). In this study, we demonstrated that TMEPAI also inhibits myostatin, activin A, activin B and GDF11 signaling, revealing that TMEPAI operates as a general inhibitor of SMAD2/3-activating ligands in muscle. Overexpression of TMEPAI specifically in skeletal muscle enhanced muscle mass by increasing fiber size and abrogated muscle atrophy induced by overexpression of activin A. Moreover, TMEPAI expression attenuated muscle wasting in an established mouse model of cancer cachexia.

In muscles overexpressing activin A, TMEPAI inhibits SMAD3 (and potentially SMAD2) phosphorylation and reduces the activin transcriptional response, as evident by a significant

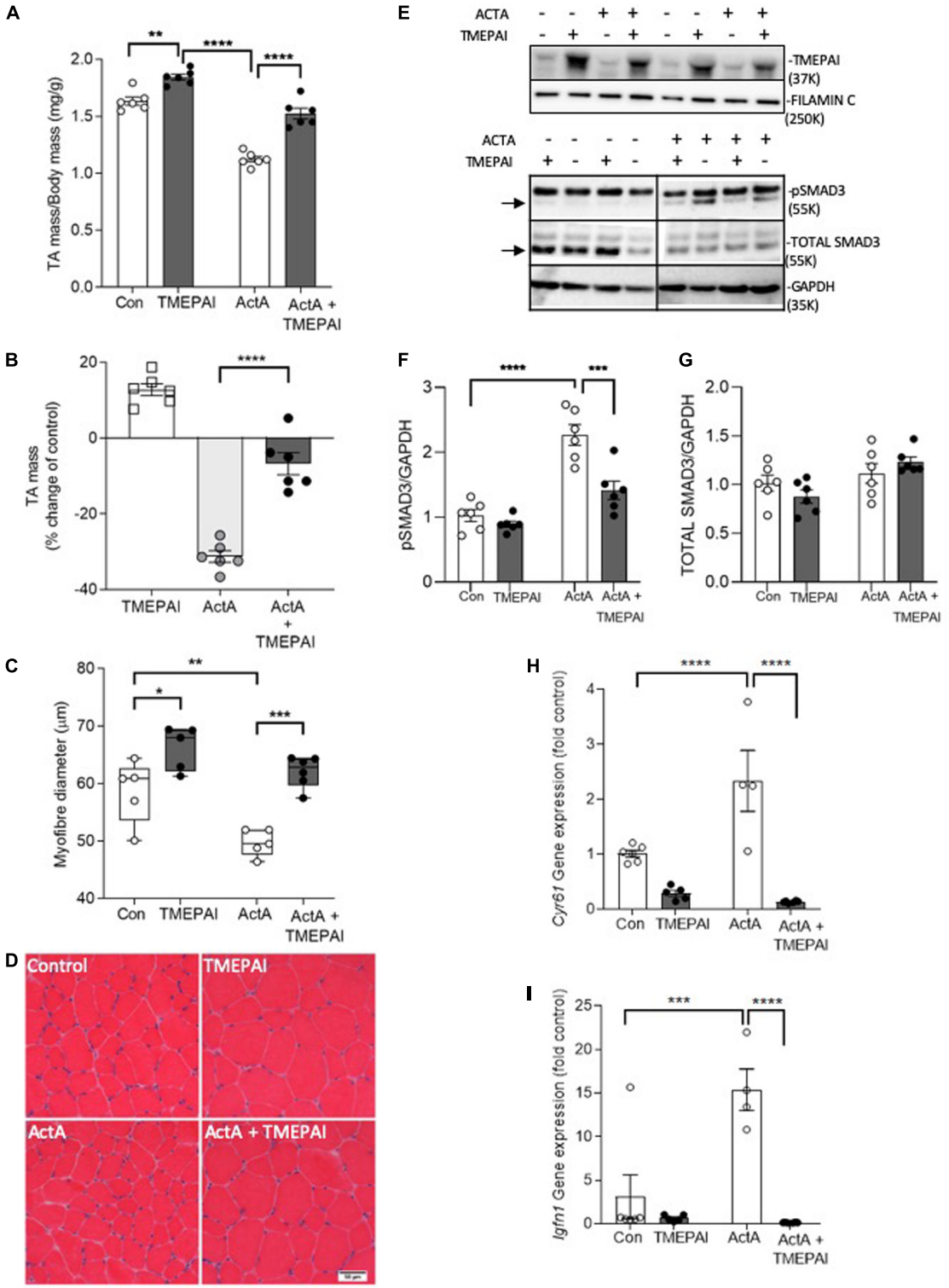

FIGURE 2 | TMEPAI promotes skeletal muscle hypertrophy and blocks activin A-mediated muscle wasting *in vivo*. **(A, B)** 4 weeks after the TA muscles of 6–8-week-old C57Bl/6J were injected with AAV vectors encoding TMEPAI and/or activin A (or equivalent doses of AAV lacking the transgene), muscles were excised to measure mass (n = 6 samples per treatment condition). **(C, D)** Mean muscle fiber diameter and representative morphology according to haematoxylin and eosin staining (n = 5 samples per treatment condition). **(E)** Protein lysates of muscles were subjected to western blot analysis of intracellular target pSMAD3 and **(F, G)** protein abundance was quantified using densitometric methods (n = 6 samples per treatment condition). **(H, I)** Expression of SMAD2/3-target genes *Cyr61* and *Igfn1* in muscles (n = 4–6 per treatment group). ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$.

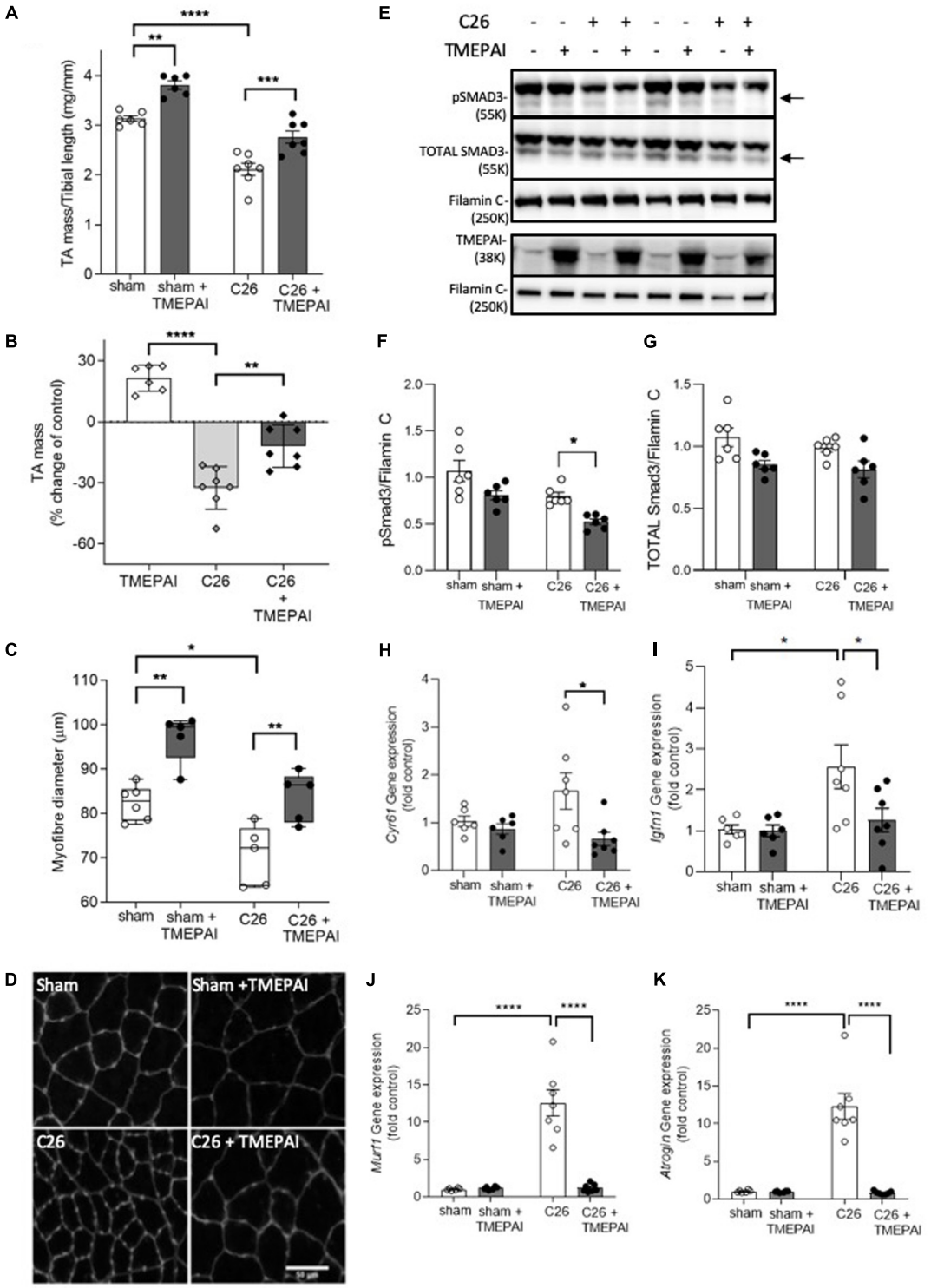

FIGURE 3 | TMEPAI prevents localized muscle wasting in a mouse model of cancer cachexia. **(A, B)** 7-week-old BALB/c mice implanted with subcutaneous Colon-26 tumors (or subjected to sham surgery) were administered TA muscle injections of AAV vector expressing TMEPAI (or control vector). At experimental endpoint, TA muscles were harvested and weighed (n = 6–7 samples per treatment group). **(C, D)** Muscle fiber size and representative morphology indicated by wheat germ agglutinin labeling of TA muscle cryosections (n = 5 samples per treatment group). **(E)** Western blot analysis of muscle protein lysates for assessment of SMAD3 phosphorylation, and **(F, G)** protein abundance quantified by densitometry (n = 6–7 samples per treatment group). **(H, I)** The transcription of SMAD2/3-target genes and **(J,K)** key muscle atrophy-related genes in the muscles from the aforementioned mice (n = 6–7 samples per treatment group). ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$.

decrease in the expression of activin-responsive genes (e.g., *Cyr61* and *Igfn1*). Similarly, in the muscles of C26 tumor-bearing mice, where activin A serum concentrations are increased (Chen et al., 2016b), SMAD3 phosphorylation is significantly reduced by TMEPAI. In support, expression of SMAD2/3 target genes *Cyr61* and *Igfn1* is also reduced in the muscles of C26 tumor-bearing mice in response to TMEPAI expression. Further, in tumor-bearing mice, TMEPAI expression suppressed transcriptional activation of muscle atrophy genes, *Murf-1* and *Atrogin-1*, likely contributing to its protective effects against muscle wasting. However, as TMEPAI can also activate SMAD-independent AR and PTEN/Akt signaling pathways (Itoh and Itoh, 2018), it is possible that additional mechanisms may contribute to TMEPAI-mediated muscle hypertrophy in these mice. Mechanistically, we showed that TMEPAI exerted its inhibitory activity via a short SIM domain, which lies within its C-terminal cytoplasmic region. Targeted disruption of the SIM domain inhibited the ability of TMEPAI to block activin A, activin B, myostatin and GDF11 activity. Conversely, the flanking PY motifs were dispensable for inhibition of SMAD2/3 signaling. This is important because the PY motifs mediate the interaction of TMEPAI with other signaling pathways (Watanabe et al., 2010) and suggest that the TMEPAI (PY) variant could be utilized to specifically inhibit SMAD2/3 signaling in skeletal muscle.

The data presented here demonstrate that TMEPAI gene delivery to skeletal muscle can attenuate activin signaling and preserve or increase muscle mass. As inhibition of activin signaling has also been shown to positively regulate metabolic processes (Han et al., 2019; Davey et al., 2020), expression of TMEPAI may also confer benefits in settings of metabolic disease. The signaling actions of TMEPAI within muscle fibers suggest that a muscle-restricted intervention could avoid the off-target effects of extracellular ligand traps, such as soluble ActRIIB. The findings support future studies to explore the extent to which systemically administered interventions that increase TMEPAI expression in skeletal muscles can protect against body-wide muscle wasting in different disease settings.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal studies were reviewed and approved by the Alfred Medical and Education Precinct Animals Ethics Committee (AMREP AEC), and the University of Melbourne Animal Ethics Committee.

AUTHOR CONTRIBUTIONS

AH, PG, CH, and KW designed the experiments. AH, SK, GG, CG, JC, RT, and HQ undertook the experiments. AH, PG, CH, and KW analyzed the data. AH, PG, CH, and KW prepared the manuscript. All authors had the opportunity to review the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.560225/full#supplementary-material

Supplementary Figure 1 | TMEPAI expression in activin A expressing muscles. **(A)** TMEPAI (*Pmepa1*) gene expression was measured in muscles previously injected with AAV:ActivinA (n = 5–6), $^{**}p < 0.01$.

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Inflammation and Skeletal Muscle Wasting During Cachexia

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Cachexia is the involuntary loss of muscle and adipose tissue that strongly affects mortality and treatment efficacy in patients with cancer or chronic inflammatory disease. Currently, no specific treatments or interventions are available for patients developing this disorder. Given the well-documented involvement of pro-inflammatory cytokines in muscle and fat metabolism in physiological responses and in the pathophysiology of chronic inflammatory disease and cancer, considerable interest has revolved around their role in mediating cachexia. This has been supported by association studies that report increased levels of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) in some, but not all, cancers and in chronic inflammatory diseases such as chronic obstructive pulmonary disease (COPD) and rheumatoid arthritis (RA). In addition, preclinical studies including animal disease models have provided a substantial body of evidence implicating a causal contribution of systemic inflammation to cachexia. The presence of inflammatory cytokines can affect skeletal muscle through several direct mechanisms, relying on activation of the corresponding receptor expressed by muscle, and resulting in inhibition of muscle protein synthesis (MPS), elevation of catabolic activity through the ubiquitin-proteasomal system (UPS) and autophagy, and impairment of myogenesis. Additionally, systemic inflammatory mediators indirectly contribute to muscle wasting through dysregulation of tissue and organ systems, including GCs via the hypothalamus-pituitary-adrenal (HPA) axis, the digestive system leading to anorexia-cachexia, and alterations in liver and adipocyte behavior, which subsequently impact on muscle. Finally, myokines secreted by skeletal muscle itself in response to inflammation have been implicated as autocrine and endocrine mediators of cachexia, as well as potential modulators of this debilitating condition. While inflammation has been shown to play a pivotal role in cachexia development, further understanding how these cytokines contribute to disease progression is required to reveal biomarkers or diagnostic tools to help identify at risk patients, or enable the design of targeted therapies to prevent or delay the progression of cachexia.

Keywords: cachexia, inflammation, muscle wasting, atrophy, cancer, COPD, cytokines

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CACHEXIA IN CANCER AND CHRONIC DISEASE

Cachexia is a complex metabolic syndrome resulting in severe weight loss, anorexia, and asthenia that occurs in many types of cancer and chronic inflammatory diseases (Evans et al., 2008). The nature of this weight loss is characterized by a pronounced systemic muscle wasting and weakness and includes marked loss of fat mass. Consequently, cachexia remains a significant co-morbidity in many diseases increasing mortality, reducing quality of life, and representing a major burden on healthcare providers. However, despite its impact in human disease the mechanisms that underpin cachexia remain poorly defined. In this review, we provide an overview and explore the latest insights into the contribution of systemic inflammation in cachexia, including both direct and indirect mechanisms of inflammatory muscle wasting.

Cachexia is highly prevalent in various types of cancers, with estimates of 30% in lung and head and neck cancer, and 41–45% in pancreatic and liver cancer, respectively (Anker et al., 2019). In addition, a high prevalence of cachexia has also been described for patients with chronic illnesses, including chronic heart failure (CHF; 10–39%) (Anker et al., 1997; Valentova et al., 2020), chronic kidney disease (CKD; 30–60%) (Mak and Cheung, 2006), chronic obstructive pulmonary disease (COPD; 5–33%) (Koehler et al., 2007; Sanders et al., 2016; Kwan et al., 2019), and rheumatoid arthritis (RA; 19–32%) (Santo et al., 2018) (although RA is often debated as being a cachectic disease as weight loss is not always a defining feature; Roubenoff, 2009). Cachexia is associated with decreased survival and quality of life in these conditions (Anker and Sharma, 2002; Reid et al., 2010; McDonald et al., 2019), and poor clinical outcome as is illustrated by increased postoperative mortality for cancer (Pausch et al., 2012) and CHF (Abel et al., 1976) and decreased response to radiation-, chemo-, and immunotherapy in the presence of cachexia (Sealy et al., 2020).

Diagnostic criteria for cachexia include weight loss in the presence of underlying illness of $>5\%$ in ≤ 12 months, or weight loss $>2\%$ in individuals with a low body-mass index (<20 kg/m^2) or low muscle mass (Fearon et al., 2011), and the presence of decreased muscle strength, fatigue or anorexia, and abnormal biochemistry (Evans et al., 2008). Interestingly, the latter refers to the presence of increased inflammatory serum markers like interleukin-6 (IL-6) and C-reactive protein (CRP), suggesting inflammation as a shared characteristic of cachexia in these distinct conditions. Indeed, several clinical studies have reported elevated levels of inflammatory markers in cancer patients with cachexia. In non-small cell lung carcinoma, CRP, IL-6, and interleukin 8 (IL-8) serum levels were increased in cachectic compared to non-cachectic lung cancer patients (Op den Kamp et al., 2013). Riccardi et al. (2020) recently highlighted changes in plasma concentrations of markers of systemic inflammation in patients with cancer cachexia compared to weight-stable cancer patients. A positive correlation between pro-inflammatory cytokines and fatty acid lipid profile was reported in patients with gastrointestinal cancer cachexia, accompanied with augmented levels of CRP, and elevated levels of pro-inflammatory cytokines IL-6, tumor necrosis factor-alpha (TNF-α), and IL-8 (Riccardi et al., 2020). In patients with gastro-esophageal cancer, multiple regression analysis identified dietary intake and serum CRP concentrations as independent variables in determining the degree of weight loss, with a higher predicted effect than disease stage (Deans et al., 2009). Based on these and other studies, it is now recognized that a systemic inflammatory response is associated with weight and muscle loss and poorer outcomes in patients with cancer (Arends et al., 2017), and can be applied in identifying the various stages of cachexia (Douglas and McMillan, 2014). A recent systematic review by Abbass et al. (2019) revealed a consistent correlation between systemic inflammation and low skeletal muscle index determined through CT and DEXA scans in patients with various types of cancer, further implicating a link between inflammation and skeletal muscle mass loss in cancer cachexia.

Similarly, a correlation between cachexia and inflammation has also been described in chronic diseases. Cachectic patients with CHF often exhibit elevated levels of pro-inflammatory cytokines such as TNF-α, IL-6, interleukin-1 (IL-1), and interferon-γ (IFN-γ), in addition to glucocorticoids (GCs) (Anker et al., 1999). These molecules act as signaling ligands to directly and indirectly impact muscle and adipose tissue metabolism in favor of wasting (Jackman and Kandarian, 2004). Patients with cardiac cachexia also exhibit elevated levels of Angiotensin II (Ang II), which has been shown to increase the levels of these pro-inflammatory cytokines (Brink et al., 2001; Zhang et al., 2009), and induce cachexia (Brink et al., 2001). In CKD patients, increased expression of TNF-α and IL-6 is detected in skeletal muscle (Zhang et al., 2020), and cachexia is accompanied by increases in circulating TNF-α and IL-6 in addition to CRP (Stenvinkel et al., 2005), which often occur in the presence of malnutrition, and therefore is referred to as malnutrition-inflammation-cachexia syndrome (Kalantar-Zadeh, 2005). In patients with CKD, cachexia was not only associated with elevated levels of CRP, but also increased fibrinogen, and reduced cross-sectional area (CSA) of muscle fibers and fat mass (Zhang et al., 2013). Cachexia development in COPD has also been associated with the presence of systemic inflammation. COPD patients with unintentional weight loss and skeletal muscle mass loss showed elevated serum cytokine levels including TNF-α and IL-6 compared to stable-weight patients (Di Francia et al., 1994; Eid et al., 2001). Disease-specific characteristics of COPD have been implicated as the driver of inflammation, such as increased TNF-α levels as a result of hypoxemia resulting from reduced lung function (Takabatake et al., 2000), or increased systemic inflammation secondary to disease exacerbations in COPD (Wedzicha et al., 2000). Respiratory infections and subsequent pulmonary inflammation are a frequent cause of exacerbation, and these episodes are accompanied by catabolic changes in skeletal muscle (Crul et al., 2010), and considered as a potentially accelerating phase in COPD cachexia (Abdulai et al., 2018). It is well established that patients with RA have elevated levels of inflammatory cytokines, specifically TNF-α, IL-1β, and IL-6, and are found directly at the synovial joint or found to be released into the plasma,

stimulating both local and systemic inflammation (Saxne et al., 1988; Shingu et al., 1993; Chikanza et al., 1995). The latter have been implicated in rheumatoid cachexia, with reports of reduced lean body mass (LBM) correlating with elevated serum levels of IL-6 and CRP (Munro and Capell, 1997; Engvall et al., 2008) In other chronic inflammatory conditions than RA, like Crohn's disease, correlations with weight loss have also been described (Nakashima et al., 1998; Bossola et al., 2000; Mantovani et al., 2001; Dulger et al., 2004; Tas et al., 2005; Baker et al., 2016), indicating that the relation between systemic inflammation and cachexia may extend beyond the diseases described here. Importantly, a correlation between circulating inflammatory cytokines and cachexia may not be detected in all patients, as a result of the rapid systemic clearance, or the presence of additional triggers of muscle and weight loss in these complex conditions, such as malnutrition in CDK (Mak and Cheung, 2006) and COPD (Creutzberg et al., 2003), or hypoxemia in COPD (Ceelen et al., 2014). In addition, exacerbations in conditions like RA, COPD, and Crohn's disease activity may be associated with transient increases in systemic inflammation, followed by sustained loss of body and muscle mass, which are not recovered as a result of other disease-related impairments in, e.g., inactivity, hypoxemia, or malnutrition (Ceelen et al., 2014).

The observed correlation between inflammation with cachexia across the various diseases described above is the basis for including serum markers of increased inflammation as a criterion in the definition of cachexia. Importantly, beyond its use as a clinical hallmark, inflammation has been investigated as a potential driver of cachexia, fueling a number of studies investigating the causal involvement of inflammation and underlying mechanisms by which it contributes to cachexia. An overview of these reports is presented below. To facilitate their discussion, a description of the processes that govern muscle mass is provided first.

Cellular Processes That Determine Muscle Mass

Maintenance and modulation of skeletal muscle mass has been attributed to two processes: protein turnover and myonuclear turnover. Under normal conditions, these processes are maintained in homeostasis, however during skeletal muscle wasting the balance within these processes shift in favor of muscle wasting, through the inhibition of muscle protein synthesis (MPS), activation of muscle protein degradation, reduction in myonuclear accretion, or increased myonuclear loss. Protein turnover is a dynamic process determined by protein synthesis and degradation and is mediated through transcriptional, translational, and post-translational mechanisms (Booth et al., 1998). One important regulatory circuit of protein turnover is the insulin growth factor-1(IGF-1)–phosphoinositide−3−kinase (PI3K)–Akt/protein kinase B (PKB)–mammalian target of rapamycin (mTOR) pathway. Akt is the key mediator stimulating protein synthesis through mTOR activation, while inhibiting protein degradation through phosphorylation of transcription factor (TF) Forkhead box O (FoxO), leading to its cytoplasmic retention where it is inactive (Schiaffino et al., 2013). Translational capacity in skeletal muscle is regulated through eukaryotic initiation factors (eIFs) and ribosomal S6 kinase (P70S6K). Formation of the eIF4F complex is a rate-limiting step in initiation of the mRNA translation process (Gingras et al., 1999), while phosphorylation of P70S6K facilitates ribosomal biogenesis and translation (Hay and Sonenberg, 2004).

Skeletal muscle is the largest latent reservoir of amino acids, which are mobilized by increased proteolysis of mainly muscle contractile proteins to provide energy or precursors for protein synthesis to other vital organs (Attaix et al., 2005). Muscle protein degradation increases as a physiological response to starvation but is also activated during pathological catabolic states that accompany inflammation and cachexia. Control of muscle mass is highly regulated by proteolytic enzyme systems including the ubiquitin-proteasomal system (UPS), autophagy-lysosomal pathway (ALP), caspases, and calpains. The UPS is involved in the removal of specific proteins for degradation following marking with ubiquitin through specific a sequential process catalyzed by ubiquitin-activating enzymes (E1), conjugating enzymes (E2), and ligating enzymes (E3) (Sorensen, 2018). It is an enzymatic process initiated by E1 enzymes activating the ubiquitin which is transferred to the E2 ubiquitin-conjugating enzyme (Ciechanover, 2005). The E2 ubiquitin complex binds to E3 protein ligases that recognize substrate proteins that will be ubiquitinated. Polyubiquitinated proteins are transferred in an ATP-dependent manner to 26S proteasome complexes in which they are degraded. E3 Ub ligases are postulated as rate-limiting factors in this pathway (Scicchitano et al., 2018), and a number of muscle enriched E3 Ub-ligases have been described, including Atrogin-1 and MuRF1. The ALP is essential for removal of misfolded or aggregated proteins and damaged parts of the cell to prevent accumulation of toxic or abnormal organelles and proteins. In addition, it enables the breakdown of proteins to produce amino acids by skeletal muscle that can be utilized in other tissues during catabolic periods, such as starvation (Sandri, 2010). There are three types of autophagy described in mammals; macroautophagy, microautophagy, and chaperone-mediated autophagy. Although all three processes are distinct from one another, these mechanisms ultimately lead to lysosomal degradation of cargo and recycling of breakdown products (Bonaldo and Sandri, 2013). Calpains and caspases are families of cysteine proteases, and their proteolytic activity is increased during cellular necrosis or apoptosis. They have been implicated in suspending cell function through disabling signal transduction molecules by enzymatic cleavage at specific amino acid residues in a host of cells (Wang, 2000), but their overall relevance to increased muscle proteolysis in muscle atrophy is not yet clear. As will be highlighted in subsequent sections, both decreases in MPS and increases in proteolysis have been implicated in the loss of muscle mass in cachexia.

Myonuclear turnover is another important process involved in muscle homeostasis and is the balance between myonuclear accretion and apoptosis. Myonuclear accretion is the final step in post-natal myogenesis (Schiaffino et al., 2013), and relies on satellite cells (SCs), the local precursor cells of skeletal muscle

(Pallafacchina et al., 2013). During muscle regeneration, SCs are activated and proliferate, which occurs through asymmetric cell division, resulting in two distinct myoblast populations. A portion of these myoblasts returns into quiescence to prevent depletion of precursor cells. The other population of myoblasts terminally differentiate and fuse with muscle fibers (or myotubes *in vitro*). Committed SCs highly express PAX7 and MYF5, which decreases during differentiation. MyoD is an important myogenic regulator during proliferation and early differentiation. Conversely, MyoG is most important in late differentiation, fusion and myotube formation (Bentzinger et al., 2012). Changes in the intricate regulation of SC activation, proliferation, and differentiation by triggers of muscle wasting, including inflammation, may result in impaired post-natal myogenesis, contributing to muscle atrophy. Myonuclear accretion seems to be impaired in cachexia as shown in animal studies (Ceelen et al., 2018a). In addition, in cachectic mice a reduced proliferation and differentiation capability of SCs was reported, resulting in myofibers not being able to regenerate or maintain their myofiber size by myonuclear accretion leading to atrophy (Inaba et al., 2018).

Evidence for a role of inflammation as a driver of cachexia and underlying mechanisms have mainly been collected in experimental models, including cultured skeletal muscle cells, animals exposed to controlled inflammatory conditions, and rodents in which diseases associated with cachexia are modeled. The marked benefit of these models is they can be deployed in experimental designs to address inflammation as a causative factor of cachexia. Moreover, they are instrumental to dissect direct and indirect effects of inflammation contributing to muscle atrophy, and provide fundamental insight in the underlying mechanisms, including the signaling pathways activated by inflammation and intra-cellular processes that cause muscle to atrophy. From these perspectives, the literature investigating the role of systemic inflammation in cachexia is discussed below.

Associations Between Systemic Inflammation and Cachexia in Disease Models

Various experimental disease models have provided incremental associative evidence to imply inflammation in the etiology of muscle wasting. In murine cancer cachexia models, the concurrent presence of systemic inflammation and loss of muscle and fat tissue has extensively been document. In Walker-256 tumor-bearing rats increased systemic inflammation was observed with animals presenting elevated plasma TNF-α and IL-6 compared to non-tumor-bearing control animals (Cella et al., 2020). These also showed increased gene expression and protein levels of MuRF1 and Atrogin-1 in hind limb muscles (Cella et al., 2020). Similar observations were reported in an orthotopic mouse model of bladder cancer, in which increased levels of inflammatory cytokines TNF-α, IL-6, and IL-1β, and activation of pro-inflammatory pathways including NF-κB were detected in muscles of urothelial tumor-bearing animals. These observations were paralleled by downregulation of Akt- and FoxO3 phosphorylation levels, suggestive of a shift toward catabolic signaling (Chen et al., 2016). Zhuang et al. (2016) showed that Atrogin-1 and MuRF1 levels were significantly increased in colon-26 (C26)-bearing mice suffering from cachexia, along with significant increases in circulating and muscle TNF-α and IL-6, whereas expression levels of anabolic IGF-1 were decreased. A recent study by Chiappalupi et al. (2020) showed that body and muscle weight loss in mice with subcutaneously growing Lewis Lung Carcinoma (LLC) cells, was accompanied by elevated serum cytokine levels, including IL-1β, IL-6, IL-10, IFN-γ, and TNF-α. Interestingly, this study highlighted the importance of receptor for advanced glycation end-products (RAGE) in sustaining the inflammatory response in tumor-bearing mice, with RAGE null mice protected against increased systemic inflammation, body weight loss, and muscle weight loss (Chiappalupi et al., 2020).

The association between inflammation and cachexia has also been extensively explored in non-cancer, inflammatory models. Jepson et al. (1986) showed administration of LPS in fed rats resulted in inflammation and reduced muscle weights accompanied by decreased MPS and increased proteolysis of skeletal muscle. This study also showed fasted untreated animals had reduced MPS, yet fasting combined with LPS treatment exacerbated this reduction, therefore highlighting that reduced food intake alone in response to LPS was not the sole cause of skeletal muscle loss (Jepson et al., 1986). Therefore, these results show that the anorectic effects of LPS alone do not fully account for the muscle atrophy observed in this model, and therefore suggest anorexia-independent effects of inflammation in muscle wasting (Jepson et al., 1986). In line, Langen et al. (2012) showed animals with LPS-induced pulmonary and systemic inflammation exhibited more skeletal muscle atrophy than pair-fed control mice, implying an additional effect of inflammation beyond anorexia or hypophagia. Similarly, LPS administered to rats to induce sepsis resulted in reduced body weights and muscle weights which could not be fully attributed to hypophagia. Here, inflammation was implicated as the primary diver of skeletal muscle loss independently of anorexia (Macallan et al., 1996). Mice with LPS and pepsin aspiration pneumonia-induced inflammation showed increased levels of IL-1β, IL-6, and MCP-1 in diaphragm and limb muscles in combination with reduced myofiber size (Komatsu et al., 2018). In this model, increased levels of MuRF1 and Atrogin-1 in muscles were shown to be indicative of elevated UPS activity and calpain and caspase-3 pathway activation (Komatsu et al., 2018). Langen et al. (2012) showed pulmonary inflammation by intratracheal instillation of LPS in mice resulted in a rapid increase in circulating pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, and CXCL1, which preceded the loss of skeletal muscle mass in these animals. In both models of pulmonary inflammation, besides evidence for UPS-mediated proteolysis, increased Bnip3, LC3B, and Gabarapl1 expression levels were measured in skeletal muscle, suggesting elevated ALP activity in lung inflammation-induced muscle atrophy (Komatsu et al., 2018). Similar findings were reported in murine models of CHF. Here cardiomyopathy, characterized by increased pro-inflammatory macrophages infiltration in cardiac muscle and elevated serum IL-6 levels resulted in elevated skeletal muscle

TNF-α and CXCR4 expression and reduced fiber CSA, indicative of inflammatory myopathy (Lynch et al., 2017; Song et al., 2019). Furthermore, CHF was shown to reduce skeletal muscle regeneration following muscle damage (Song et al., 2019).

Despite the diversity in primary pathology, and the degree and kinetics by which cachexia develops in these disease models, systemic inflammation and its preceding and correlation with muscle wasting appear a consistent factor beyond simple association in cachexia.

(Pre)clinical Evidence for Inflammation as a Cause of Cachexia in Disease

Several *in vivo* studies have highlighted the importance of specific pro-inflammatory cytokines or activation of specific inflammatory pathways in muscle wasting through genetic or pharmaceutical inhibition, showing causal evidence that inflammation is a required component of atrophy in pathological models.

In models of cancer cachexia, e.g., mice or rats with methylcholanthrene-induced sarcoma (MCG-101) or in LLC tumor bearing mice, skeletal muscle wasting was alleviated through the blockade of the apex pro-inflammatory cytokine TNF-α through administration of anti-TNF antibodies (Sherry et al., 1989; Torelli et al., 1999). In addition, intra-muscularly inoculated LLC-tumor bearing WT mice displayed increased protein degradation and loss of muscle mass, which was prevented in transgenic littermates overexpressing the soluble TNF receptor type 1 protein (sTNF-R1) to inhibit the actions of local or circulating TNF-α (Llovera et al., 1998). While this study implies a causal contribution of TNF-α in skeletal muscle atrophy, this may result from indirect effects of TNF-α by increasing other cytokines such as IL-6 that contribute to cachexia development by impacting on skeletal muscle. Indeed, IL-6 has been implicated in cancer cachexia. $Apc^{Min/+}$ mice with cancer cachexia showed significant muscle wasting in presence of a 10-fold increase of serum IL-6 levels compared to control groups (Baltgalvis et al., 2009). In addition, host-IL-6 was shown to be required in the development of cachexia in these mice, with $Apc^{Min/+}$/IL-$6^{-/-}$ mice showing reduced tumor burden and muscle wasting (Baltgalvis et al., 2008). Furthermore, administration of anti-murine IL−6 receptor antibody to C−26−bearing mice attenuated muscle wasting, in support of IL-6-dependent muscle atrophy in colon cancer cachexia (Fujita et al., 1996).

Administration of both anti-TNF-α and an IL1-receptor antagonists in models of cancer cachexia also shows evidence of preservation of body weight and improved food intake compared to untreated tumor-bearing controls, suggesting a common mechanism for both cytokines (Gelin et al., 1991). Furthermore, anti-TNF-α and anti-IL1-R treatment also reduced tumor growth, suggesting indirect effects of inflammation as a determinant of tumor burden in driving atrophy (Gelin et al., 1991). Similarly, in a rat RA model, administration of soluble TNF receptor I (sTNFRI) as a TNF blocking strategy improved body weight, but also food intake compared to control groups, implying that anti-inflammatory modulation may contribute to bodyweight maintenance by blocking anorexic effects (Granado et al., 2006). In line with that notion, in a cardiac cachexia rodent model, anti-TNF-α treatment significantly reduced losses in body and skeletal muscle mass, partly through reduced UPS activation through the attenuation of anorexia (Steffen et al., 2008). Finally, in a model of cachexia induced by *Trypanosoma cruzi*, mice treated with anti-TNF-α antibodies displayed significant attenuation of weight loss, while anti-IL-6 and anti-IFN-γ antibodies had no such effect. In addition, this protection of weight loss occurred during the acute phases of infection and was only transient in nature, suggesting early administration of anti-TNF therapies may be more effective in the early phases of cachexia (Truyens et al., 1995).

The concept of a causal role of pro-inflammatory cytokines mediating cachexia in humans has only been addressed very limitedly and only in a few pathological conditions, using targeted therapeutics that deplete specific cytokines such as TNF-α and IL-6. In contrast to the experimental models, the effects of TNF-α blockade in cancer cachexia, while complicated by the diversity of disease etiologies, and limitations due to the actions of the corresponding treatments on tumor immunity, have proven less promising. Here a number of studies examining different cancer patient cohorts have failed to identify any meaningful changes in body weights, LBM, or muscular function (determined by 6 min walk test) in response to anti-TNF-α interventions (Jatoi et al., 2007, 2010; Wiedenmann et al., 2008; Gueta et al., 2010). Studies examining anti-IL-6 in cachexia are limited. One case report in a patient with large-cell carcinoma of the lung and cancer cachexia demonstrated improved inflammatory outcomes with reduced serum IL-6 after prednisolone treatment, with no further deterioration in cachexia parameters (Ando et al., 2013). While these studies are frequently complicated by poor accrual, recruitment, and interactions with other disease-related complications and between ongoing therapeutic interventions, thus far the causal involvement of inflammation in muscle atrophy during cancer cachexia has been difficult to assess in clinical studies.

In COPD, several TNF blocking agents have reached phase-II clinical trials, but these have been complicated by initial concerns related to increased incidence of cancer compared to the placebo control treatment arms (Rennard et al., 2007), which were later contested in a long-term follow-up analysis (Rennard et al., 2013). While the original rationale for anti-TNF treatment was to intervene in the lung pathology, more recent insights suggest that specific groups of patients, in particular COPD patients with cachexia may benefit from TNF-blocking agents (Rennard et al., 2013). Studies appropriately designed to assess this remain to be initiated, however. In contrast, TNF-α blockade has proven highly effective in the management of chronic inflammatory disease such as RA (Saxne et al., 1988). It must be noted that in RA, evaluating the direct contribution of inflammation on muscle mass and function is complicated by the actions of these treatments on disease activity, which reduce pain and allow for improvements in dietary intake and physical activity. Here, several such studies failed to report meaningful correlations in changes in body composition, in response to anti-TNF-α treatments over short

durations (Marcora et al., 2006; Elkan et al., 2009). These studies are complicated by side by side comparison with disease modifying anti-rheumatic drugs such as methotrexate, which possess their own anti-inflammatory immunomodulatory effects that may mask muscle protective actions. However, studies examining anti-TNF-α intervention in RA over longer periods revealed improvement in body weight, BMI, total and fat mass relative to patients receiving standard disease management treatment (Chen et al., 2013; Toussirot et al., 2014). Moreover, promising results have been observed in alternative chronic inflammatory arthropathies and inflammatory diseases such as ankylosing spondylitis and Crohn's disease. Here, in ankylosing spondylitis, improvement in muscle strength parameters was reported following anti-TNF-α intervention, while increases in both muscle volume and strength were evident in patients with Crohn's (Subramaniam et al., 2015; Demirkapi et al., 2017).

Despite the ambiguous evidence from clinical studies, the preclinical disease models strongly support a causal relationship between inflammation and cachexia. The extent to which actions of inflammation depend on interactions with other pathology-related alterations, or whether inflammation *per se* is sufficient to induce cachexia, is addressed in different experimental models described next.

Causal Evidence Implying Inflammation as a Driver of Skeletal Muscle Atrophy

Many *in vivo* studies have shown that the induction of an inflammatory state by TNF-α infusion initiate the development of cachexia, resulting in reduced food intake, loss of bodyweight, and skeletal muscle loss (Tracey et al., 1988; Llovera et al., 1993). In addition, implantation of a continuously TNF-α producing tumor cell line into mice, elicited cachexia and weight loss, with reduced food intake, compared to the control, non-secreting tumor cell line (Oliff et al., 1987). TNF-α has also shown to suppress the IGF-1 pathway and cause insulin resistance, which may also play a role in the dysregulation of macronutrient uptake and utilization (Broussard et al., 2003; Frost et al., 2003). Mice inoculated with tumors that overexpress IFN-γ presented with severe cachexia, and IFN-γ inhibition prior to inoculation attenuated body weight loss (Matthys et al., 1991), implying IFN-γ secretion rather than other tumor-dependent effects in the development of tissue depletion in this model. In mice inoculated with tumor cells expressing Fn14, a receptor for the inflammatory cytokine tumor necrosis factor-like weak inducer of apoptosis (TWEAK), significant cachexia development and reduced survival rates were observed (Johnston et al., 2015). Moreover, anti-Fn14 monoclonal antibodies prevented cachexia development, while tumor growth rate being reduced, implying local or reciprocal effects of anti-Fn14 on tumor growth and cachexia (Johnston et al., 2015). In line, chronic administration or muscle-specific transgenic overexpression of TWEAK in mice resulted in reduced body and skeletal muscle weight with an associated increased activity of UPS and NF-κB (Dogra et al., 2007).

Alternatively, the release of pro-inflammatory cytokines is triggered in models of sepsis. Schakman et al. (2012) induced inflammation in rats through LPS injection, which lead to a loss of body and muscle weight. IGF-1 levels were significantly reduced, and accompanied by an upregulation of FoxO1, Atrogin-1, and MuRF1. In a similar model, identical findings were reported, as well as increased muscle TNF-α expression (Dehoux et al., 2003), indicative of local inflammatory signaling and activation of proteolysis in this model of muscle wasting. Increased levels of systemic inflammatory cytokines may result from spill over from an inflamed site, as is the cause for pulmonary inflammation. Ceelen et al. (2018b) evoked pulmonary inflammation in mice, which resulted in systemic inflammation and muscle atrophy, with accompanying of UPS-mediated proteolysis and upregulation of E3 ligases MuRF1 and Atrogin-1. Interestingly, there were no additive effects of body weight loss and muscle wasting in emphysematous mice compared to control groups after LPS exposure, and can therefore be concluded muscle atrophy was a direct consequence of the pulmonary inflammation and not affected by the presence of emphysema (Ceelen et al., 2017). This group also showed evoking repetitive pulmonary inflammation in emphysematous mice, mimicking recurrent acute exacerbations in COPD, resulted in sustained muscle atrophy, which was associated with markers of impaired muscle regeneration, with altered myogenic signaling and reduced fusion capacity (Ceelen et al., 2018a). In line, chronic pulmonary inflammation in transgenic mice with lung-specific overexpression of TNF resulted in muscle atrophy and an impaired muscle regenerative response compared to WT littermate control animals (Langen et al., 2006).

Taken together, these studies demonstrate inflammation is sufficient to drive muscle wasting beyond the context of disease-induced cachexia. However, these models cannot distinguish direct from indirect effects, i.e., requiring involvement of another tissue or intermediary paracrine or autocrine signal, impacting on the intramuscular processes that drive muscle mass loss in cachexia. As such understanding is instrumental for development of intervention strategies, an extensive number of studies focused on identifying the intracellular pathways responsible for sensing inflammatory signals and transducing these into atrophy responses.

DIRECT EFFECTS OF INFLAMMATION: SIGNALING PATHWAYS AND ACTIVATING LIGANDS RESPONSIBLE FOR RELAYING DIRECT, MUSCLE ATROPHY-INDUCING EFFECTS OF INFLAMMATION

The direct effects of inflammation on skeletal muscle require receptor-mediated activation of intra-muscular signaling pathways. Various signaling pathways activated by inflammatory cytokines, or inflammation-associated ligands have been

implicated in muscle atrophy through regulation of muscle protein turnover or myonuclear turnover. These pathways and the corresponding activating ligands (overview in **Figure 1**) that have been implicated in muscle atrophy are described in this section, including their impact on muscle protein and myonuclear turnover.

NF-κB-Signaling

Nuclear factor-κB (NF-κB) is a TF, and activation of classical NF-κB signaling occurs in response to various inflammatory cytokines (e.g., IL-1 and TNF-α) and oxidative stress. The former act through corresponding receptor binding and activation, and recruitment of adaptor proteins, resulting in I-kappa-B kinase (IKK) activation and culminating in NF-κB nuclear translocation. TNF receptor-associated factors (TRAF) is a family of intracellular adaptor proteins that interact with the surface receptors TNFR-1 and -2, Toll-like receptor 4 (TLR4), and IL-1R. TNF-α receptor adaptor protein 6 (TRAF6) not only integrates upstream inflammatory signals, but is central to the activation of many signaling pathways including NF-κB and MAPK in response to cytokines (Paul et al., 2010; Miao et al., 2017). Activity if this adaptor protein is elevated in cachectic LLC-bearing mice, while TRAF6 depletion attenuated muscle wasting in tumor bearing mice (Paul et al., 2010). Downstream, NF-κB activation has been implicated as an important step in inflammation-induced muscle wasting. Inhibition of NF−κB alleviates the cytokine-driven atrophy of muscle, thus highlighting NF-κB in the direct effects of inflammatory stimuli in muscle wasting (Ladner et al., 2003). Prevention of muscle NF-κB activation in genetically modified mice attenuated muscle wasting in a model of pulmonary inflammation-induced systemic inflammation (Langen et al., 2012). Similarly, both, pharmacological (Miao et al., 2017) and muscle-specific genetic (Cai et al., 2004) inhibition of NF-κB prevented muscle wasting in tumor bearing mice, indicating the importance of this TF in cancer cachexia. Conversely, muscle specific activation of the NF−κB pathway in transgenic mice resulted in profound muscle atrophy (Cai et al., 2004).

NF-κB activation has been implicated in increasing UPS proteolytic activity through the expression of E3 Ub-ligases genes Atrogin-1 and MuRF1 (Kandarian and Jackman, 2005; Dillon et al., 2007; Schiaffino et al., 2013). In lung inflammation-induced muscle atrophy, genetic inhibition of skeletal NF−κB inhibited the increases in MuRF1 expression (Langen et al., 2012), which was shown as a required step for muscle wasting in a similar model using MuRF1 knock-out (KO) mice (Files et al., 2012). In line, upregulation of MuRF1 was also required for muscle atrophy observed in response to muscle specific activation of the NF−κB pathway in transgenic mice (Cai et al., 2004). Furthermore, NF-κB, through Akt inhibition, leads to elevated FoxO activity which stimulates the expression of UPS- and ALP-related genes, such as LC3 and Bnip3 (Hanna et al., 2012). Additionally, NF-κB prevents myoblast cell cycle exit, reduces MyoD and Myf-5 protein abundance and activity (Langen et al., 2004), and decreases MyoD mRNA expression (Guttridge et al., 2000), leading to impaired post-natal myogenesis (Guttridge et al., 2000; Langen et al., 2001, 2004). Furthermore, *in vitro* and *in vivo* studies have also shown that serum factors from cachectic mice and patients, in an NF-κB-dependent manner, induce expression of the self-renewing factor Pax7, implying NF-kB inhibits myogenic differentiation through sustained Pax7 expression (He et al., 2013).

Muscle wasting-inducing properties of the inflammatory cytokines TNF-α and IL-1 have mainly been attributed to receptor-mediated activation of NF-κB, and involve increased proteolysis as well as impaired myogenesis, Several *in vitro* studies have shown inflammatory cytokine TNF-α administration in C2C12 myocytes leads to activation of NF−κB signaling (Guttridge et al., 2000; Langen et al., 2001). The NF−κB inhibitor PDTC inhibited upregulation of MuRF1 induced by TNF-α medium *in vitro* through inhibition of NF−κB indicating the importance of this signaling pathway. TNF-α induced upregulation of the catabolic genes Atrogin-1 and MuRF1 parallel to inducing myotube atrophy in differentiated C2C12 cells (Li et al., 2005). In L6 and C2C12 myotubes treated with TNF-α, decreased eIF3f translation initiation factor abundance and increased Atrogin-1 levels were observed during myotube atrophy, suggestive of decreased protein synthesis and elevated proteolysis, respectively (De Larichaudy et al., 2012). Genetic inhibition of NF-κB was also reported to prevent TNF-induced myotube atrophy (Liu et al., 2010), although in later work by this group TNF-α was postulated to act via p38 to increase Atrogin-1 and MuRF1 (Li et al., 2005), which was confirmed in another study for TNF-α-induced Atrogin-1 in C2C12 myotubes (Chiappalupi et al., 2020). IL-1 is an inflammatory cytokine which actions overlap with TNF-α, and can be elevated during cancer cachexia (Cederholm et al., 1997). C2C12 incubation with either IL-1α or IL-1β resulted in reduced myotube size and activation of NF-κB signaling, in turn leading to increased Atrogin-1 and MuRF1 expression (Li et al., 2009). Another activator of NF-kB signaling implicated in muscle atrophy concerns the cytokine TWEAK and its receptor fibroblast growth factor inducible 14 (Fn14). TWEAK has been shown to be capable of inducing inflammation, which was reduced in Fn14-deficient mice, through unknown mechanisms, implying TWEAK as a feed-forward signal for an inflammatory state (Girgenrath et al., 2006). Following TWEAK binding to Fn14 it can activate various signaling modules through its adaptor proteins (e.g., TRAF6), leading to NF-κB and MAPK activation (Sato et al., 2014). Myotubes incubated with TWEAK show increased NF-κB activation (Dogra et al., 2007; Bhatnagar and Kumar, 2012), MuRF1 and ALP-related genes such as Beclin1, and activation of caspases. Inhibition of MuRF1, autophagy, or caspase-3 blocked the TWEAK-induced degradation of MyHC and myotube atrophy (Bhatnagar et al., 2012). Furthermore, TWEAK incubation *in vitro* can inhibit Akt phosphorylation, leading to reduced protein synthesis while stimulating protein degradation (Dogra et al., 2007).

In vivo, TNF-α overexpression has been shown to impair proliferative and myogenic responses during muscle regeneration (Langen et al., 2006). In differentiating C2C12 myocytes, activation of NF-κB by TNF-α incubation lead to the inhibition of MyoD through destabilization of MyoD mRNA (Guttridge et al., 2000) and MyoD protein (Langen et al., 2004). Another

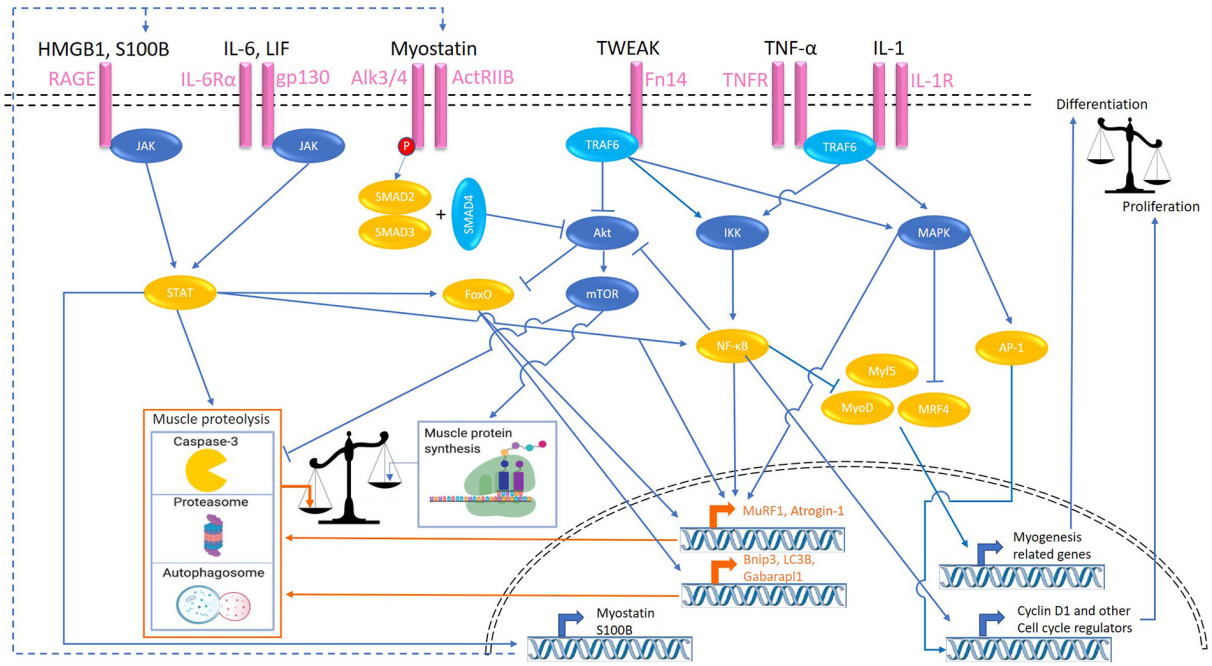

FIGURE 1 | Signaling pathways activated by inflammatory ligands involved in cachexia-related muscle atrophy. Colors refer to transcription factors (orange), proteolytic signaling (orange), kinases (dark blue), adaptor proteins (light blue), and cell surface receptors (pink). HMGB1, high mobility group box 1; S100B, S100 calcium-binding protein B; RAGE, receptor for advanced glycation endproducts; ActRIIB, activin receptor type IIB; TWEAK, tumor necrosis factor-like weak inducer of apoptosis; Fn14, fibroblast growth factor-inducible 14; TNFα, tumor necrosis factor-α; IL, interleukin; JAK, Janus kinase; STAT, signal transducers and activators of transcription; TRAF, TNF receptor associated factor; FoxO, Forkhead box transcription factors; mTOR, mammalian target of rapamycin; IKK, IκB kinase; NF-κB, nuclear factor-κB; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; MyoD, myoblast determination protein 1; MyoG, myogenin; MRF4, myogenic regulatory factor; AP-1, activator protein 1; MuRF-1, muscle RING-finger protein-1; Bnip3, BCL2 interacting protein 3; Gabarapl1, GABA type A receptor associated protein like 1.

study showed that when using the NF−κB inhibitor PDTC the induction of atrogenes which may have contributed to MyoD and MyHC proteolysis was inhibited in cells incubated with TNF-α (Miao et al., 2017). TNF-α has been shown to stimulate myoblast proliferation at the expense of differentiation *in vitro* in an NF-κB-dependent manner (Otis et al., 2014). Similarly, it was also shown IL-1 induces proliferation which was inhibited after inhibiting NF-κB, indicating this effect is NF-κB mediated (Otis et al., 2014). Two other studies also showed IL-1 stimulated NF-κB activity *in vitro*, which also showed increases in proliferation of both primary and C2C12 myoblasts, highlighting IL-1 impacts on myogenic activity in skeletal muscle cells (Langen et al., 2001; Otis et al., 2014). TWEAK has also been shown to convey anti-differentiation and pro-proliferation actions through inducing sustained NF-κB activation and MyoD degradation in addition to reduced expression levels of MyoD and MyoG *in vitro* (Dogra et al., 2006; Girgenrath et al., 2006; Winkles, 2008).

Besides direct atrophy-inducing effects of TNF signaling through NF-kB, autocrine activation of parallel pathways in muscle have also been described. Treatment of TNF-α alone or combined with IFN-γ increased the expression of RAGE and its ligands S100B, and HMGB1 in C2C12 myotubes (Chiappalupi et al., 2020). Subsequent atrophy of myotubes and increased Atrogin-1 and MuRF1 mRNA expression levels required the presence of RAGE, and involved JAK-STAT activation, implying an autocrine signaling circuit downstream of TNF-induced p38 MAPK and NF-κB activity.

JAK/STAT-Signaling

The JAK/STAT pathway is activated by type I (IFN-α/β), type II (IFN-γ), IL-2, and IL-6 receptor stimulation (Schindler et al., 2007). IL-6 binding to the IL-6r-Gp130 receptor complex results in the recruitment to the intracellular domain of the receptor, and subsequent activation of the JAK tyrosine kinase. After binding, JAK proteins undergo a conformational change, dimerize, and activate the STAT proteins through phosphorylation. Subsequently homo- or hetero-dimerization of STAT proteins is followed by translocation to the nucleus (Moresi et al., 2019). STAT transcriptional activation contributes to muscle wasting through various mechanisms. It stimulates CCAAT/enhancer binding protein (C/EBPδ) expression and activity, which in turn increases myostatin, MAFbx/Atrogin-1, MuRF1, and caspase-3 expression in myofibers (Haddad et al., 2005; Zhang et al., 2013; Silva et al., 2015), enhancing muscle proteolysis. Moreover, increased myostatin expression resulting from STAT-C/EBPδ activation suppresses post-natal myogenesis (Zhang et al., 2013), which in turn may negatively affect muscle mass maintenance. Furthermore, STAT was documented to regulate gene transcription by interaction with FoxO and NF-κB (Oh et al., 2012; Yoon et al., 2012).

Interleukin-6 is a pleiotropic cytokine which can induce several intra-cellular signaling pathways including JAK/STAT in a variety of cells types. Intra-cellular signaling through the binding of IL-6 to the IL-6R in turn associates with the transmembrane protein Gp130, which is ubiquitously expressed in most cells. The soluble form of IL-6R (sIL-6R) is found in most bodily fluids and also binds to IL-6, further increasing the range of target tissues for IL-6 as the IL-6-sIL-6R complex has the ability to bind and activate to Gp130 on any cell, this is known as "trans- signaling" (Heinrich et al., 2003). IL-6 has been implicated as a core mediator of cancer cachexia. Indeed, systemic IL-6 concentrations increase with intestinal tumor development in $Apc^{Min/+}$ mice and is associated with elevated p-STAT-3 and Atrogin-1 mRNA levels (Baltgalvis et al., 2009). In addition, host-IL-6 was shown to be required in the development of cachexia in these mice, with $Apc^{Min/+}$/IL-$6^{-/-}$ mice showing reduced tumor burden and muscle wasting (Baltgalvis et al., 2008). In line, increased circulating IL-6 levels and elevated STAT-3 signaling were detected in skeletal muscle of C26 cachectic mice, and inhibition of STAT-3 attenuated muscle atrophy *in vitro* and *in vivo* (Bonetto et al., 2012). Blockade of IL-6R through administration of an anti-murine IL-6R antibody in C26-bearing mice also showed attenuated muscle loss and reduced expression of cathepsin B and L in muscle compared to tumor-bearing controls (Fujita et al., 1996), highlighting the requirement of IL-6 and IL-6 signaling in this experimental model of cancer cachexia. Conversely, overexpression of human IL-6 increased expression of proteasomal subunits cathepsins B and L in muscle and induced muscle atrophy in transgenic mice (Tsujinaka et al., 1995), highlighting that chronic elevation of circulating IL-6 is sufficient to cause muscle wasting. In support of a role for increased proteolysis, intra-peritoneal injections of IL-6 lead to increased muscle atrophy in rats measured by tyrosine and 3-methylhistidine release (Goodman, 1994). In addition, reduced phosphorylation of PS6K1, indicative of reduced translational capacity, and protein synthesis have been reported in muscles infused with IL-6 (Haddad et al., 2005). Local IL-6 infusion into the TA muscle decreased total and myofibrillar protein content in rats (Haddad et al., 2005), suggesting that atrophy-inducing effects of IL-6 are the result of direct actions of IL-6 on skeletal muscle. In support of this, C2C12 myotubes treated with recombinant IL-6 did show reduced myotube diameter, reduced mTOR and 4EBP-1 phosphorylation, and increased STAT3 phosphorylation and Atrogin-1 transcription, showing IL-6 suppresses mTOR and therefore reducing protein synthesis, in addition to increasing atrogene expression (White et al., 2013). However, other studies have shown little effect of IL-6 on skeletal muscle both *in vivo* and *in vitro* (García-Martínez et al., 1994; Ebisui et al., 1995), which may be explained by differences in IL-6 levels, shorter exposure regiments, or the pleiotropic nature of IL-6 in skeletal muscle, e.g., the source of IL-6 (Daou, 2020).

Gp130 has been implicated as the main cellular receptor in skeletal muscle to mediate the IL-6 effects in cancer cachexia. Mice injected with LLC with a genetic deletion of Gp130 specifically in skeletal muscle showed attenuated muscle wasting compared to WT controls, primarily through reduced STAT signaling and atrogin-1 and FoxO3 activation (Puppa et al., 2014). In line, hyperactivation of STAT3 signaling through Gp130 activation in $gp130^{F/F}$ knock-in mice with a k-Ras-driven lung carcinoma developed cachexia with reduced muscle and fat mass and reduced life expectancy compared to k-Ras mice without Gp130 hyperactivation (Miller et al., 2017). Although these results show the importance of the activation of Gp130 and STAT signaling in cancer cachexia, Gp130 activation is not solely restricted to IL-6.

Leukemia inhibitory factor (LIF) has also recently been identified as a cytokine, which activates the same receptor as IL-6 and also mediates skeletal muscle atrophy through STAT and ERK signaling (Seto et al., 2015). Seto et al. showed in a murine model of C26 colon carcinoma with muscle atrophy, serum levels of LIF increased in parallel to tumor development. LIF was actively secreted by C26 tumor cells, whereas TNF-α and IL-6 were not, and incubation of C2C12 myotubes with LIF was sufficient to induce atrophy. Conversely, LIF inhibition in tumor cell conditioned media (CM) prevented CM-induced myotube atrophy *in vitro*, while genetic inactivation of STAT3 in myofibers was sufficient to suppress atrophy *in vivo* (Seto et al., 2015). RAGE is part of the immunoglobulin superfamily and known as a key mediator of several pathological processes. It is activated by ligands including high mobility group box 1 (HMGB1) and the S100 calcium−binding protein B (S100B), which are secreted by various cell types, including tumor cells and damaged myofibers (Chiappalupi et al., 2020). Furthermore, inflammatory cytokines activate a feed-forward RAGE signaling loop by inducing HMGB1, S100B, and RAGE expression in skeletal muscles (Chiappalupi et al., 2020). Its potential relevance to cancer cachexia is suggested by increased serum levels of S100B and HMGB1 in the serum of cancer patients (Miyamoto et al., 2016; Chiappalupi et al., 2020), and highlighted by the observation that LLC-bearing RAGE/KO mice displayed delayed body and muscle weight loss, reduced Atrogin-1 and MuRF1 expression levels, and prolonged survival time compared to WT mice. The cytosolic domain of RAGE connects to JAK/STAT3 signaling, implicated in increased protein degradation and decreased differentiation, but has also been reported to activate the tyrosine kinase protein, Src, which is implicated in several other downstream signaling hubs, such as ERK1/2, p38 MAPK, JNK, and NF-κB (Riuzzi et al., 2018).

MAPK Signaling

The MAPK pathway controls growth and stress responses in a myriad of cell types, including skeletal muscle. MAPK signaling is activated by cellular stress, growth factors, and pro-inflammatory cytokines (e.g., IL-1 and TNF-α) (Zhang and Liu, 2002). The MAPK family of proteins consists of four distinct signaling pathways, namely, extracellular signal-regulated kinases 1 and 2 (ERK1/2), p38 MAPK, c-Jun NH2-terminal kinases (JNK), and ERK5 (Kramer and Goodyear, 2007). p38 MAPK mediates upregulation of MuRF1 and Atrogin-1 in response to TNF-α by an unknown mechanism (Li et al., 2005; Chiappalupi et al., 2020). IL-1 signaling has also been shown to stimulate phosphorylation of p38 MAPK, leading to increased

atrogin-1 expression, independent of Akt/FoxO signaling (Li et al., 2009). Furthermore, p38 phosphorylates MRF4, thus inhibiting the expression of selective myogenic genes in late myogenesis, and antagonizes the JNK proliferation-promoting pathway (Suelves et al., 2004; Perdiguero et al., 2007). JNK mediates AP-1 activation, which is a signaling molecule that controls proliferation and differentiation through transcriptional regulation of cell-cycle regulators such as cyclin D1, cyclin A, and cyclin E (Kyaw et al., 2002; Hess et al., 2004), and has been implicated in muscle atrophy responses (Liu et al., 2010). When treated with TNF-α, C2C12 increased p-ERK in differentiating myoblasts, which correlated with suppressed MyoD and MyoG levels, and reduced accretion of myosin heavy chain content. Administration of the ERK inhibitor PD98059 to C2C12 cells prevented this inhibitory effect of TNF-α on myogenic differentiation (Penna et al., 2010).

SMAD-Signaling

The smad pathway is activated by multiple ligands, but in the context of muscle mass control, Myostatin/GDF8, a member of the transforming growth factor-β (TGF-β) family, is the best described (Lee and Jun, 2019), next to GDF11 (Egerman et al., 2015) and Activin-A (Trendelenburg et al., 2012). Myostatin is a myokine, and its autocrine and paracrine effects act as a break on skeletal muscle growth. Myostatin has been found to be associated with cancer cachexia and its expression is stimulated through the JAK/STAT pathway (Costelli et al., 2008; Zhang et al., 2013). This positions Smad signaling secondary to transcriptional activation of Mstn by inflammatory cues. Binding of myostatin to ActRIIB results in the phosphorylation of Smad2/3 (El Shafey et al., 2016) and activation of Smad signaling, which reduces p-Akt levels (Trendelenburg et al., 2009), consequently activating caspase-3 and FoxO, and resulting in increased protein degradation (Schiaffino et al., 2013; Zhang et al., 2013). Accordingly, downregulation of p-Akt and p-FoxO3 accompanied by myostatin and activin A overproduction in the muscle were seen in mice with bladder cancer (Chen et al., 2016), implying myostatin involvement in cachexia triggered by various cancer types (Zimmers et al., 2002; Chen et al., 2016). Myostatin administration is sufficient to induce cachexia in mice through ActRIIB signaling (Costelli et al., 2008). Conversely, blockade of the ActRIIB receptor prevented cachexia in C26 tumor bearing mice, without affecting increased circulating levels of IL-6, TNF-α, and IL-1β (Zhou et al., 2010), implying ActRIIB signaling acts independent, or downstream of inflammation-associated muscle atrophy through autocrine expression of ActRIIB activating ligands like myostatin or Activin-A (Trendelenburg et al., 2012). In support of this notion, inhibition of myoblast differentiation by inflammatory cytokines was found to require *de novo* Activin-A production (Trendelenburg et al., 2012), implying smad signaling secondary to an autocrine mechanism activated by inflammation.

Combined, these studies identify a myriad of inflammatory cytokines and ligands as mediators of inflammation, which directly impact on skeletal muscle through receptor-mediated signaling which affects muscle protein turnover in favor of proteolysis or impairs myogenesis, ultimately resulting in muscle wasting.

INDIRECT EFFECTS OF INFLAMMATION

In addition to the direct effects inflammatory cytokines induced by receptor-mediated activation of signaling pathways in skeletal muscle, cytokines also cause dysregulation of other tissue and organ systems which indirectly contribute to muscle wasting and cachexia development. As such, dysregulation of the hypothalamus-pituitary-adrenal (HPA) axis and adrenocorticoids, anorexia and malnutrition, changes in adipocyte behavior, and hepatic metabolism have shown to impact cachexia progression (see overview in **Figure 2**).

Involvement of the HPA Axis and Corticosteroid Synthesis in Cachexia

Glucocorticoids such as cortisol are endogenous pleotropic hormones that play an essential role in glucose mobilization and energy metabolism, as well as having potent anti-inflammatory immune modulatory actions (Barnes, 1998). In the 1950s, Kendall, Reichstein and Hench received a Nobel prize for their work in the isolation and application of the GCs in the treatment of chronic inflammatory diseases such as RA. Unfortunately, the clinical efficacy of endogenous and synthetically derived GCs was tempered by severe metabolic side effects, including osteoporosis, truncal obesity, and muscle wasting. The mechanisms underpinning GC induced muscle wasting are compound, with evidence of reduced anabolic IGF-1 signaling and increased anti-anabolic myostatin production, resulting in a reduction in the mTOR signaling pathway, as well as induction of catabolic UPS and autophagy mediated muscle protein degradation, secondary to increased FOXO1 signaling (Gayan-Ramirez et al., 1999; Imae et al., 2003; Fenton et al., 2019), and decreased myogenesis (Pansters et al., 2013).

Due to their muscle atrophy-inducing actions, considerable interest exists regarding possible dysregulation of endogenous steroid synthesis in conditions such as chronic inflammation and cancer cachexia. In particular, the HPA axis is a central regulatory target activated in response to systemic inflammation and stress that has been widely investigated (Ulrich-Lai et al., 2006; Engeland et al., 2016). This critical homeostatic regulatory pathway mediates the synthesis and release of the endogenous GC hormone, cortisol, from the zona fasciculata of the adrenal cortex (Silverman and Sternberg, 2012). Classically, the HPA axis is under central circadian regulation by the hypothalamus, where it drives a pulsatile diurnal secretion of cortisol through the release of corticotropin-releasing hormone (CRH) from the paraventricular nucleus. This in turn results in the systemic release of adrenocorticotropin (ACTH) from the anterior pituitary, which binds to the MC2R receptor in the adrenal cortex to initiate adrenal cortisol synthesis and release (Silverman and Sternberg, 2012; Ruggiero and Lalli, 2016). During inflammation, pro-inflammatory factors such as TNF-α, IL-1β, and IL-6 can act at all levels of the

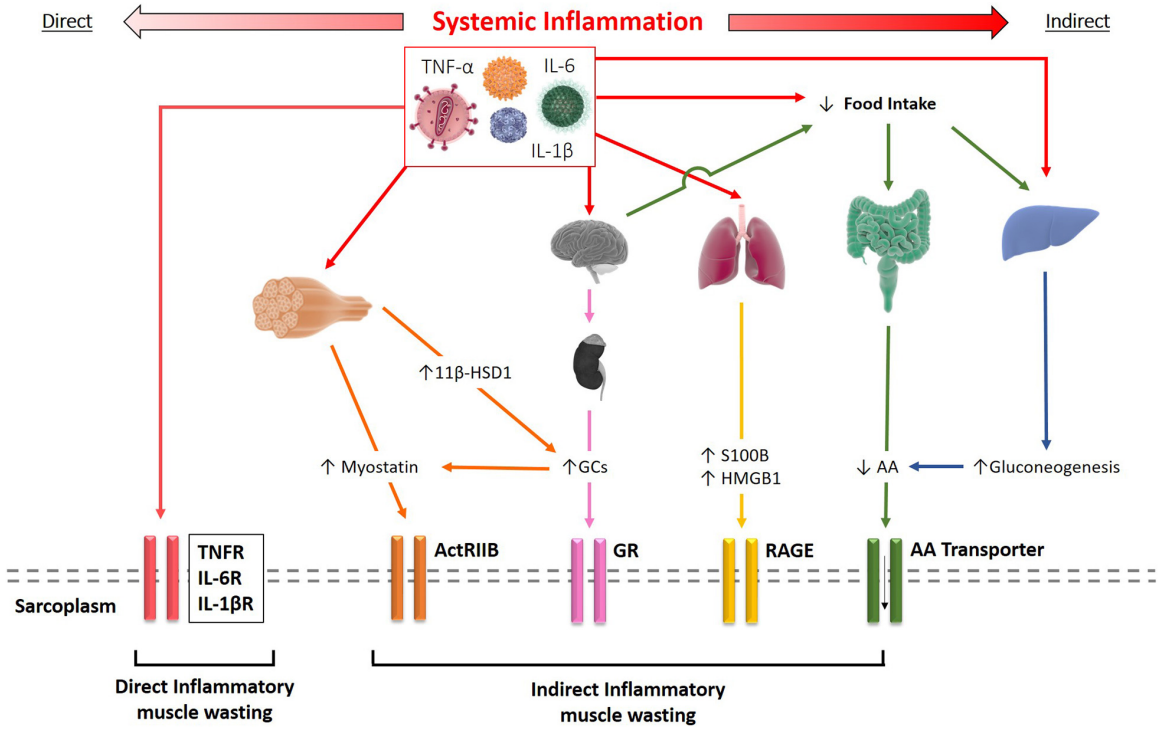

FIGURE 2 | Schematic representation of the direct and indirect effects of systemic inflammation resulting in muscle wasting. Inflammatory cytokines such as TNF-α and IL-1β can bind to their receptors on the sarcolemma driving skeletal muscle wasting directly. Alternatively, cytokines may elicit their effects indirectly through several mechanisms, including increased myostatin and glucocorticoid signaling, release of S100B and HMGB1 by inflamed tissue, or reduced amino acid (AA) availability.

HPA axis to increase CRH, ACTH from the hypothalamus and pituitary, and cortisol synthesis and release from the adrenals (Mastorakos et al., 1993; Nolten et al., 1993; Curti et al., 1996). Conversely, negative regulation of this pathway is achieved through the direct suppression of CRF and ACTH by cortisol.

In particular, focus has fallen upon the concept that either inflammatory cytokines, or disease treatments (such as chemotherapy in cancer) (Braun et al., 2014), cause dysregulation of central hypothalamic/pituitary negative feedback resulting in the over activation of the HPA axis in cachexia (Braun et al., 2011), leading to circulating steroid excess and GC induced muscle wasting as well as loss of adipose tissue (Crofford, 2002; Russell and Tisdale, 2005). In addition, the mixed immunomodulatory actions that circulating GCs may mediate in cancer immunity and in suppression of pro-inflammatory cytokines may also influence disease progression and cachexia. However, studies exploring the dysregulation of the HPA axis specifically in cachexia have yielded mixed results. In mice, several studies report increased activity of the HPA axis in models of cancer cachexia and COPD, coinciding with weight loss (Russell and Tisdale, 2005; Crespigio et al., 2016; Flint et al., 2016; de Theije et al., 2018). de Theije et al. (2018) showed that hypoxia-induced muscle wasting in a murine model of COPD was in turn dependant on GC signaling with GC receptor/KO mice being partly protected from muscle wasting. Several studies in cancer patients (including colorectal, prostate, and breast) report dysregulation of the HPA axis with increased levels of serum cortisol (Strassmann et al., 1992; Soygur et al., 2007; Fearon et al., 2013; Flint et al., 2016). In several of these instances, increases in serum GCs were linked with elevated levels of the pro-inflammatory cytokine IL-6, suggesting this may be a key mediator of increased HPA axis activity. However, whether these changes regulated cachexia in addition to influencing tumor immunity was not fully elucidated (Flint et al., 2016). In addition, muscle GC signaling was found to be required for cancer-induced cachexia (Braun et al., 2013), and muscle atrophy in response to inflammation-evoking cytotoxic chemotherapy was shown to depend on intact GC signaling in skeletal muscle (Braun et al., 2014). Similarly, muscle-specific deletion of GR prevented endotoxin-induced muscle atrophy (Braun et al., 2013). In line, LPS injections in rats induced an inflammatory response, body weight loss, and muscle wasting including upregulation of FoxO and other atrogenes (Schakman et al., 2012). In contrast to TNF-α and NF−κB inhibitors, only inhibition of the GC receptor using RU-486 blunted LPS-induced atrogene expression in this model, highlighting the importance increased GC signaling in inflammation-associated muscle wasting (Schakman et al., 2012). Consequently, the true nature of HPA axis and steroid dysregulation in cachexia, while of significant interest, have proven hard to fully elucidate and show significant disease specific variation.

A Role for Pre-receptor Steroid Metabolism in Glucocorticoid-Induced Muscle Atrophy

While the systemic regulation of circulating endogenous GC levels is determined through the HPA axis, peripheral exposure to GCs is mediated through their tissue specific pre-receptor metabolism. This is primarily mediated by the 11beta-hydroxysteroid dehydrogenase (11β-HSD) enzymes types 1 and 2 (Hardy et al., 2014). Here, 11β-HSD1 primarily mediates the peripheral conversion of the inactive GC precursor cortisone, to its active counterpart cortisol (11-DHC to corticosterone in rodents) within target tissues, where it greatly amplifies local GC signaling. In contrast, 11β-HSD2 solely inactivates GCs, converting active cortisol to cortisone (corticosterone to 11-DHC in rodents) blocking local GC signaling. 11β-HSD1 shows a diverse pattern of expression across a wide array of tissues including liver, fat, muscle, bone, and in immune cells (Tomlinson et al., 2002; Hardy et al., 2008, 2016). In contrast, 11β-HSD2 expression appears to be limited to tissues such as the kidney where it protects against inappropriate activation of the mineralocorticoid receptor by GCs (Quinkler et al., 2005). Renewed interest in the roles of 11β-HSD1 in inflammatory muscle wasting and cachexia have been fueled by observations that its expression and GC activation are potently upregulated in peripheral tissues such as muscle in response to pro-inflammatory factors such as IL-1β and TNF-α (Ahasan et al., 2012; Hardy et al., 2016). These studies raised the possibility that under conditions of chronic inflammation, local amplification of GC signaling by 11β-HSD1 may represent a critical component in mediating inflammatory muscle wasting. This concept was lent further credit following a seminal study by Morgan et al. (2014) demonstrating that the systemic transgenic deletion of 11β-HSD1 in murine models of GC excess completely abrogated GC induced muscle wasting. However, the only study to examine the role of inflammatory 11β-HSD1 in muscle revealed a complex interplay between the anti-inflammatory actions of GCs versus their anti-anabolic catabolic actions (Hardy et al., 2016). Here, while the transgenic deletion of 11β-HSD1 in murine models of inflammatory polyarthritis resulted in reduced GC signaling in muscle, the exacerbation of muscle inflammation drove a more florid muscle wasting phenotype. Consequently, the role of 11β-HSD1 in other forms of muscle wasting and cachexia requires further investigation.

Inflammation-Driven Anorexia and Muscle Wasting in Cachexia

Cachexia development is profoundly impacted by the accompaniment of anorexia, categorized by reduced appetite and nutritional deficit which ultimately leads to catabolism of lean body and adipose tissue (Bosaeus et al., 2002). Anorexia–cachexia is distinct from starvation, where skeletal muscle loss is less apparent compared to adipose tissue. Adipose tissue is a reservoir for energy, and therefore in times of starvation or reduced energy intake, catabolism of adipose tissue allows the release of energy which is then used in processes that maintain skeletal muscle mass, however, in cachectic patients both muscle and fat tissue are catabolized as energy sources. In addition, nutritional interventions alone are unable to reverse or alleviate this catabolic phenotype (Moley et al., 1987; Thomas, 2002; von Haehling and Anker, 2010). Although the pathogenesis of anorexia–cachexia is multifactorial, inflammatory cytokines have been shown to be implicated in the development of anorexia in cachectic patients through an amalgamation of mechanisms (Laviano et al., 2003). In some disease states, such as cancer, tumor burden has been implicated in driving anorexia–cachexia through dysphagia or dysregulation of gastro-intestinal function, ultimately leading to reduced food intake and nutritional deficit (Ezeoke and Morley, 2015).

Rats receiving a single dose of human TNF-α resulted in increased muscle proteolysis and anorexia (Bodnar et al., 1989; Flores et al., 1989), while tumor bearing rats receiving TNF-α inhibitors had markedly improved nutritional intake and body weights (Torelli et al., 1999). These findings suggest TNF-α indeed plays a pivotal role in inducing anorexia, although its full contribution the development of cachexia is yet to be elucidated. Plata-Salamán et al. (1988) demonstrated TNF-α administration in rodents suppressed food intake in a dose-dependent manner, through the cytokine directly acting on glucose-sensitive neurons in the central nervous system (CNS) to suppress appetite. Lung cancer patients exhibiting anorexia showed reduced hypothalamic activity compared to non-anorexic patients; however, circulating levels of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1 were not significantly different between groups (Molfino et al., 2017). Another cytokine implicated in anorexia–cachexia is IL-1, with several studies observing its effects on food intake. These anorexia-inducing effects of IL-1 have been illustrated in several *in vivo* studies, with both peripheral and central administration decreasing food intake in rodents (Kent et al., 1994). Furthermore, Layé et al. (2000) previously showed increases in IL-1 in the hypothalamus in rodents upon LPS administration and reduced food intake, and IL-1 antagonists preventing LPS-induced anorexia. However, many of these *in vivo* studies also show development for tolerance to cytokines, and therefore the interpretation of results is often debated (Mrosovsky et al., 1989). Another study highlights the importance of central regulation of appetite in response to inflammation, with GLP-1 receptor antagonist mitigating anorexia induced by LPS in rats (Grill et al., 2004).

Peripheral hormones that directly affect nutrition status through central actions controlling appetite have also been shown to play an important role in anorexia–cachexia. Ghrelin, a peptide released in the gut shown to stimulate appetite, is decreased in response to acute inflammation (Basa et al., 2003; Otero et al., 2004). In contrast, chronic inflammation in animal models and cachectic patients present with increased ghrelin levels (Nagaya et al., 2001a; Shimizu et al., 2003; Dixit et al., 2004), possibly a compensatory effect of ghrelin resistance in cachectic and catabolic states (Li et al., 2004). Mechanisms underpinning ghrelin's role in anorexia–cachexia have not yet been established; however, experimental models of cachexia have demonstrated ghrelin administration suppressed weight loss and alleviated skeletal muscle wasting through increased food intake

(Nagaya et al., 2001b; Hanada et al., 2003). Another cytokine shown to be of importance in the role of developing cachexia–anorexia is leptin, which is released from adipocytes and signals to the hypothalamus to regulate nutritional intake as a satiety cue. Leptin has been shown to be increased in rodents and humans exhibiting cachexia in many disease states, such as CHF (Cheung et al., 2005). Therefore, the role of leptin in anorexia–cachexia is not well established and is often speculated that the alterations in leptin levels may be in response to malnutrition and reduced fat mass rather than a consequence of elevated inflammatory cytokines.

While these data collectively support a role for pro-inflammatory cytokines in the development of anorexia, due to cachexia's multifactorial phenotype, it is difficult to underpin the mechanisms in which cytokines may drive anorexia–cachexia. However, a plethora of research indicates that energy deficits in combination with reduced hypothalamic response may play a pivotal role in anorexia–cachexia (Ramos et al., 2004).

Role of Inflammation-Induced Alterations in Adipocyte and Hepatic Metabolism in Cachexia

There is increasing evidence to show preserving adipose tissue in cachexia can improve mortality and quality of life (Murphy et al., 2010). Lipolysis stimulation during cachexia can be induced by anorexia; however, there is also evidence for inflammation-induced lipolysis. Reduction in food intake or starvation induces lipolysis to release energy stores; however, as lipid stores are depleted, other catabolism of tissues will ensue to provide sufficient energy, of which a main energy source is amino acids derived from skeletal muscle proteolysis (Finn and Dice, 2006). As discussed previously, inflammation can induce anorexia–cachexia, reducing food and energy intake, which ultimately leads to the reduction of fat mass and loss of white adipose tissue (WAT) through lipolysis. Cachexia is also associated with loss of skeletal muscle and WAT through increased energy expenditure (Bosaeus et al., 2001), and related to increased inflammation in pancreatic cancer patients presenting with elevated resting energy expenditure in addition to increased CRP levels (Falconer et al., 1994). Patients with cancer cachexia were shown to have increased levels of circulating IL-6 and enhanced lipolysis compared to weight-stable cancer patients, which was not attributed to enhanced locally expressed IL-6 levels, implicating not the inflammatory infiltrate but other triggers for adipose tissue wasting (Rydén et al., 2008). One mechanism that has been described to contribute to this increased energy expenditure is the remodeling of WAT into brown adipocytes, which has been suggested to occur prior to skeletal muscle wasting in cancer-cachexia (Petruzzelli et al., 2014). This increase in brown adipose tissue increases thermogenesis in these patients (Lee et al., 2010), which ultimately leads to an increased requirement for energy, and thus increased energy expenditure. Interestingly, IL-6 has been implemented in increasing uncoupling protein 1 (UCP1) expression, a protein found in brown adipose tissue that increase thermogenesis (Li et al., 2002). In addition, mice with syngeneic grafts of C26 cells lacking IL-6 showed protection against weight loss and reduced UCP1 expression compared to mice with active IL-6 C26 cells, thus highlighting the importance of this cytokine in WAT browning, and therefore increased energy expenditure in cachexia (Petruzzelli et al., 2014). However, the exact role of WAT browning in skeletal muscle loss in cachexia requires further investigation.

Although various studies have shown the effects of inflammatory cytokines on adipose tissue and skeletal muscle, only few address these in the context of cachexia. Mice s.c. injected with LLC or B16 melanoma cells showed cachexia development, with reduced body weights, WAT loss, muscle wasting, and increased serum TNF-α and IL-6 levels (Das et al., 2011). Inhibition of lipolysis through genetic deletion of adipose triglyceride lipase (Atgl), a mediator of lipolysis, in tumor-bearing animals showed protection against cachexia development, with reduced WAT and skeletal muscle loss. However, TNF-α and IL-6 levels remained increased in the serum of these animals, highlighting a possible indirect action of these cytokines in driving cachexia and adipose tissue loss through lipolytic mechanisms (Das et al., 2011). Importantly, this study also emphasizes the importance of crosstalk between adipose tissue and skeletal muscle, as inhibition of lipolysis resulted in reduced skeletal muscle wasting, therefore suggesting that altered free fatty acid or adipokine release may play a role in skeletal muscle wasting. Adipose tissue secretes adipokines, such as leptin, with endocrine functions including satiety and whole-body metabolism (Galic et al., 2010). Several inflammatory cytokines such as IL-6, TNF-α, and IL-1β are adipokines as well as myokines, and have been implicated in reciprocal control of adipose and muscle mass (Muñoz-Cánoves et al., 2013; Daas et al., 2019) and metabolism (Piya et al., 2013). Further research, however, is required to disentangle the role of inflammation in adipose and muscle reciprocal effects in the context of cachexia.

Although the liver is a central regulator of metabolism, there is relatively little research examining a role of the liver in the association between inflammation and cachexia, which is surprisingly considering the liver is the major site for muscle proteolysis-derived amino acids for utilization in gluconeogenesis and acute-phase protein synthesis, such as CRP (Argilés et al., 2001), and elevated CRP levels are the most frequently applied additional criteria to assess cachexia (Fearon et al., 2006). Indeed, profound hepatic alterations are observed prior to and during the progression of cancer cachexia, including alterations in fat metabolism, collagen deposition, and fibrosis (Rosa-Caldwell et al., 2020). In line, despite not evaluating liver-anatomical changes, alterations in liver metabolism in inflammation associated cachexia have been reported. $Apc^{min/+}$ mice with severe cachexia were shown to have increased levels of acute phase protein haptoglobin, revealing hepatic alterations in inflammation-associated cancer cachexia (Narsale et al., 2015). In a model of pancreatic cancer cachexia, inhibition of proliferator-activated receptor-alpha (PPAR-α) through IL-6 resulted in hypoketonemia and subsequent activation of the HPA axis, ultimately leading to increased GC release and enhanced muscle proteolysis (Flint et al., 2016). In addition, Goncalves et al. showed adult $Kras^{G12D/+};Lkb1^{f/f}$ (KL) mice with lung cancer and cachexia presented with increased IL-6 levels, increased gluconeogenesis in the liver, reduced hepatic fatty acid oxidation, and hypoketonemia. Skeletal muscle atrogenes MuRF1

and Atrogin-1 were upregulated and also noted a decrease in type II fiber CSA (Goncalves et al., 2018). PPAR-α inhibitor fenofibrate restored hepatic ketogenesis, which in turn reduced the requirement for the liver to use gluconeogenesis and alleviate the need for type II skeletal muscle degradation for amino acids (Goncalves et al., 2018). These results therefore show the indirect effects of both liver metabolism and GCs on skeletal muscle wasting in cachexia.

Myokines as a Nexus and Opportunity in Modulating Inflammation-Associated Cachexia

Apart from the participation of other tissues as an intermediary step between inflammation and induction of muscle wasting, a role for autocrine, paracrine, and even endocrine acting signals derived from skeletal muscle in cachexia is emerging. These concern the "myokines," e.g., cytokines, growth factors, and other peptide-based molecules released from skeletal muscle (Pedersen et al., 2003). In the context of muscle mass regulation, Mstn is a well-characterized myokine for its muscle growth-inhibitory actions. Increased expression (Kim et al., 2018) and signaling (Zhang et al., 2013) of Mstn in skeletal muscle may constitute an autocrine mechanism of Mstn-dependent muscle wasting in response to inflammatory cues. In line with this notion, blockade of the ActRIIB receptor to inhibit Mstn signaling prevented cachexia in C26 tumor bearing mice, without affecting increased circulating levels of IL-6, TNF-α, and IL-1β (Zhou et al., 2010). Interestingly, increases in muscle Mstn expression and secretion may also contribute to muscle wasting in an endocrine route in case of RA, as Mstn has been implied in inflammatory bone destruction, aggravating RA-associated muscle loss (Fennen et al., 2016). In C26 tumor-bearing BALB/c mice, involvement of myokines was further indicated as increased muscle IL-6, IL-6R, and myostatin expression accompanied muscle wasting in these mice (Lee et al., 2019). Recent work has revealed GDF15 as a myokine, which is expressed at low levels during homeostasis, but can be induced by muscle contraction (Laurens et al., 2020), or metabolic stress (Ost et al., 2020) or increased GDF11 levels (Jones et al., 2018) in skeletal muscle. GDF15 circulating levels correlate inversely with skeletal muscle mass in COPD (Patel et al., 2016), and increasing GDF15 levels are sufficient to induce dramatic weight loss (Johnen et al., 2007). As thus far no evidence supports the expression of the GDF15 receptor, GFRAL, a co-receptor of the Ret tyrosine kinase, in skeletal muscle, this suggests endocrine effects of GDF15 when secreted as a myokine. As such, muscle derived GDF15 was reported to stimulate lipolysis in adipocytes (Laurens et al., 2020), which in the context of cachexia could contribute to adipose tissue depletion. Importantly, elevation of GDF15 suppresses appetite via activation of hypothalamic neurons (Johnen et al., 2007), and the cachexia-inducing properties GDF15 are thought to be a result of anorexia (Johnen et al., 2007). Although induction of GDF15 expression by TNF-α and NF-κB regulation has been shown for other cell types (Ratnam et al., 2017), it remains to be explored whether GDF15 expression increases in skeletal muscle in response to inflammatory cytokines.

In contrast, other myokines including IL-15 (Tamura et al., 2011) and musculin (Nishizawa et al., 2004) have been attributed anabolic effects or anti-catabolic effects on skeletal muscle, at least in part mediated through autocrine mechanisms. In addition, IL-6, when secreted by skeletal muscle in response to stimuli such as exercise, exerts endocrine effects such as lipid oxidation (van Hall et al., 2003), which contribute to organismal homeostasis. Interestingly, in tumor-bearing mice, exercise was found to attenuate tumor growth, which correlated with increased IL-6 levels post-exercise, and systemic IL-6 blocking experiments revealed IL-6 may actually contribute to hindering tumor growth (Pedersen et al., 2016). Moreover, exercise-induced increases in IL−6 contribute to an anti-inflammatory systemic environment, by increasing the production of the anti-inflammatory cytokines, IL−1β receptor antagonist (IL−1ra), and IL−10 (Steensberg et al., 2003). A recent murine study suggested that IL-6 may induce either pro- or anti-inflammatory actions depending on cell source (Han et al., 2020), potentially explaining the beneficial, suppressive effects on tumor growth and immunomodulatory actions of muscle derived IL-6. In addition, C2C12 differentiating myoblasts showed increased IL-6 levels during differentiation in combination with increased STAT3 phosphorylation. Blockade of IL-6 independently showed reduced differentiation of myotubes, highlighting the crucial role IL-6 has in differentiating myotubes (Hoene et al., 2012). As such, these studies may reflect an endocrine cachexia-modulating potential of myokines.

CONCLUSION AND FUTURE PERSPECTIVES

Despite the overwhelming preclinical evidence to imply inflammation as both sufficient and required in disease-associated cachexia, this has not translated into unambiguous success of cytokine-depleting therapeutic agents to reverse cachexia in patients with cancer or chronic disease. This may reflect the complex interactions within an inflammatory response, rendering a therapy based on inhibition of a single cytokine therapy insufficient. Combined blocking approaches (Truyens et al., 1995), or downstream inhibition of molecules at which inflammatory cues convergence, like NF-κB (Miao et al., 2017) or STAT3 (Ahasan et al., 2012) have shown effective in experimental models and warrant further exploration for pharmacological modulation. In addition, the timing of anti-inflammatory treatment may be of key importance. Much of the evidence in the experimental models indicates inflammation precedes a cachectic phenotype, and anti-inflammatory interventions successfully modulating cachexia in preclinical studies are without exception started prior to initiation of cachexia development. Consequently, for anti-inflammatory agents to be effective in a clinical setting, this may require interventions to start in patients at risk for cachexia, i.e., "pre-cachectic," for subsequent evaluation of their ability to prevent or delay onset of cachexia. Bearing time in mind as an important determinant of the efficacy anti-inflammatory modulation, its downstream signaling should be further considered. Feed-forward signals that transform

inflammatory cues of systemic origin into an autocrine, muscle atrophy-promoting signal, have been reported for Mstn (Zhang et al., 2013) and Activin-A (Trendelenburg et al., 2012). Receptor blocking agents for these ligands are available, and the first clinical trials have yielded promising results in terms of safety and efficacy in COPD patients with low muscularity (Polkey et al., 2019). These ActRIIB inhibitors are continuously refined (MacDonald et al., 2012), and further improvement in their efficacy to halt or even reverse cachexia progression is anticipated when provided as an integral part of a multimodal therapy, i.e., combined with appropriate nutritional support and tailored exercise programs. Similarly, desensitizing skeletal muscle to the atrophy-inducing effects of GCs, by inhibition of local GC-activation using therapeutic 11β-HSD1 inhibitors that are currently in clinical trials for other applications (Harno and White, 2010), may be a route to explore the potential of blocking the indirect effects by which inflammation contributes to muscle wasting. Finally, skeletal muscle tissue itself may hold the key to counteracting inflammation driven cachexia, as myokines have been attributed very potent immunomodulatory features, which in future research deserve further investigation in their potential to prevent and reverse skeletal muscle wasting in cachexia.

Combined, the extensive efforts to delineate the underlying mechanisms of inflammation-associated cachexia have revealed insights that provide multiple leads to evaluate novel, more selectively targeted therapeutic approaches in this debilitating condition.

AUTHOR CONTRIBUTIONS

JW and LK systematically reviewed potentially relevant manuscripts to extract and synthesize the findings into the various sections of the review. RL conceived the focus of the manuscript. RH and RL defined the structure of the review, critically edited the content of the paragraphs, and defined conclusions. All authors contributed to the article and approved the submitted version.

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The Acute Effects of 5 Fluorouracil on Skeletal Muscle Resident and Infiltrating Immune Cells in Mice

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5 fluorouracil (5FU) has been a first-choice chemotherapy drug for several cancer types (e.g., colon, breast, head, and neck); however, its efficacy is diminished by patient acquired resistance and pervasive side effects. Leukopenia is a hallmark of 5FU; however, the impact of 5FU-induced leukopenia on healthy tissue is only becoming unearthed. Recently, skeletal muscle has been shown to be impacted by 5FU in clinical and preclinical settings and weakness and fatigue remain among the most consistent complaints in cancer patients undergoing chemotherapy. Monocytes, or more specifically macrophages, are the predominate immune cell in skeletal muscle which regulate turnover and homeostasis through removal of damaged or old materials as well as coordinate skeletal muscle repair and remodeling. Whether 5FU-induced leukopenia extends beyond circulation to impact resident and infiltrating skeletal muscle immune cells has not been examined. The purpose of the study was to examine the acute effects of 5FU on resident and infiltrating skeletal muscle monocytes and inflammatory mediators. Male C57BL/6 mice were given a physiologically translatable dose (35 mg/kg) of 5FU, or PBS, i.p. once daily for 5 days to recapitulate 1 dosing cycle. Our results demonstrate that 5FU reduced circulating leukocytes, erythrocytes, and thrombocytes while inducing significant body weight loss (>5%). Flow cytometry analysis of the skeletal muscle indicated a reduction in total CD45+ immune cells with a corresponding decrease in total CD45+CD11b+ monocytes. There was a strong relationship between circulating leukocytes and skeletal muscle CD45+ immune cells. Skeletal muscle Ly6c^{High} activated monocytes and M1-like macrophages were reduced with 5FU treatment while total M2-like CD206+CD11c- macrophages were unchanged. Interestingly, 5FU reduced bone marrow CD45+ immune cells and CD45+CD11b+ monocytes. Our results demonstrate that 5FU induced body weight loss and decreased skeletal muscle CD45+ immune cells in association with a reduction in infiltrating

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Ly6c^{High} monocytes. Interestingly, the loss of skeletal muscle immune cells occurred with bone marrow cell cycle arrest. Together our results highlight that skeletal muscle is sensitive to 5FU's off-target effects which disrupts both circulating and skeletal muscle immune cells.

Keywords: chemotherapy, monocytes, macrophages, skeletal muscle, bone marrow

BACKGROUND

The increase in 5-year survival rate among cancer patients has increased focus on quality of life to improve patient outcomes (Curt et al., 2000). In addition to cancer-associated wasting and functional decrements, the most commonly prescribed chemotherapies have pervasive off-target effects that have been reported to impact quality of life (Iacovelli et al., 2014; Lee et al., 2014; Polk et al., 2014; Ribeiro et al., 2016). 5 fluorouracil (5FU) has been the first-choice chemotherapy drug for several cancer types for many years (van Kuilenburg and Maring, 2013; Giuliani and Bonetti, 2016; Lee et al., 2016; McQuade et al., 2017); however, 5FU negatively impacts the gastro-intestinal system (Lee et al., 2014; Ribeiro et al., 2016; Sougiannis et al., 2019), cardiovascular system (Polk et al., 2014; Phillips et al., 2018), hematopoietic system (Kvinnsland, 1999; Shitara et al., 2009; Han et al., 2012), and has recently been shown to directly disrupt skeletal muscle (Barreto et al., 2016b; Botsen et al., 2018; Williams et al., 2018). Disruptions to skeletal muscle homeostasis contributes to functional dependency and poor treatment outcomes and ultimately leads to increased healthcare costs and decreased survival (Barreto et al., 2016b). Currently, there are no Food and Drug Administration approved therapies for chemotherapy-induced cachexia despite the importance of skeletal muscle and lean mass in sustaining 5FU's therapeutic efficacy and patient quality of life (Sandini et al., 2018; Williams et al., 2018). This is not entirely surprising given that very little is known about the mechanisms responsible for 5FU-induced skeletal muscle dysfunction. Thus, identifying the factors driving chemotherapy-induced skeletal muscle dysfunction is critical to developing effective interventional therapies.

Despite 5FU-induced leukopenia remaining a hallmark of treatment (Shitara et al., 2011; Sougiannis et al., 2019), investigations into the impact of 5FU on skeletal muscle have largely focused on metabolism (Barreto et al., 2016a,b). Notably, there is a dearth of evidence on the influence of 5FU on skeletal muscle inflammation – a process that is known to play a role in skeletal muscle homeostasis (Costamagna et al., 2015). Indeed, inflammation can play a paradoxical role in skeletal muscle homeostasis. During normal conditions pro-inflammatory cytokines are required to balance anabolism and catabolism and to maintain normal myogenic processes. However, during disease conditions, pro-inflammatory cytokines can induce catabolic pathways that impair skeletal muscle integrity and function (Sharma and Dabur, 2020). To date, our understanding of 5FU-induced inflammatory changes is limited to circulating inflammatory cytokines and intrinsic inflammatory signaling. Additionally, the available studies highlight equivocal results showing increased circulating interleukin (IL) 6, tumor necrosis factor α (TNFα), monocyte chemoattractant protein (MCP) 1 (Wang et al., 2012; Mahoney et al., 2013, 2014), with reduced or unchanged skeletal muscle inflammatory protein expression (Barreto et al., 2016a,b). Given the importance of skeletal muscle to quality of life in chemotherapy patients along with the well-documented effects of inflammation on skeletal muscle homeostasis, it is important to assess inflammatory mediators as a potential target for chemotherapy-induced skeletal muscle dysfunction (Kvinnsland, 1999; Yamanaka et al., 2007; Shitara et al., 2009; Baechler et al., 2010; Han et al., 2012; Abraham et al., 2015).

The lack of evidence on 5FU associated perturbations in skeletal muscle inflammation is consistent with a scarcity of literature on 5FU effects on skeletal muscle immune cells. Monocytes, or more specifically macrophages, are the most abundant skeletal muscle immune cell which function to regulate tissue turnover and homeostasis (Tidball, 2017). Targeting macrophages is emerging as a potential key regulator of chemotherapeutic efficacy given the importance of tumor associated macrophages (TAM) in tumorigenesis, tumor vascularization, and local immunosuppression (Mantovani and Allavena, 2015); however, the effects of 5FU on skeletal muscle macrophages is largely unexplored. Resident skeletal muscle monocytes are classically characterized as CD11b+Ly6c^{Low} monocytes and F4/80+CD11c-CD206- (quiescent – M0) macrophages (Krippendorf and Riley, 1993; Tidball, 2017). Circulating CD11b+Ly6C^{High} activated monocytes, recruited by MCP-1, extravasate the muscle (Deshmane et al., 2009; Liao et al., 2018) and either remain CD11b+Ly6C^{High} or differentiate to F4/80+CD11c+CD206- pro-inflammatory, pro-phagocytic (M1-like) macrophages which secrete pro-inflammatory cytokines, IL-6, IL-1β, TNFα, and interferon (IFN) γ (Frenette et al., 2003; Guilliams et al., 2014). These M1-like macrophages will then down regulate CD11c expression and increase CD206+ to reflect a more anti-inflammatory, pro-fibrotic (M2-like) macrophage which secrete anti-inflammatory cytokine IL-10 and pro-fibrotic cytokine transforming growth factor (TGF) β (Gordon and Taylor, 2005; Arnold et al., 2007; Reidy et al., 2019). Proper balance of these immune cell phenotypes and maintenance of immune cell number are vital for skeletal muscle homeostasis. Thus, determination of 5FU's effects on skeletal

Abbreviations: 5FU, 5 fluorouracil; BSA, bovine serum albumin; CD, cluster of differentiation; DMEM, Dulbecco's Modified Eagle Medium; EDTA, Ethylenediaminetetraacetic acid; FBS, fetal bovine serum; FSC, forward scatter; HCT, Hematocrit; HGB, Hemoglobin; IFN, interferon; IL, Interleukin; Ly6c, lymphocyte antigen 6c; LYM, lymphocyte; MCP, monocyte chemoattractant protein; MON, monocyte; NEU, neutrophil; PBS, phosphate buffered saline; PLT, platelets; RBC, red blood cell; SEM, standard error of the mean; SSC, side scatter; STAT, signal transducer and activator of transcription; TAM, tumor associated macrophage; TGF, transforming growth factor; TNF, tumor necrosis factor; WBC, white blood cell.

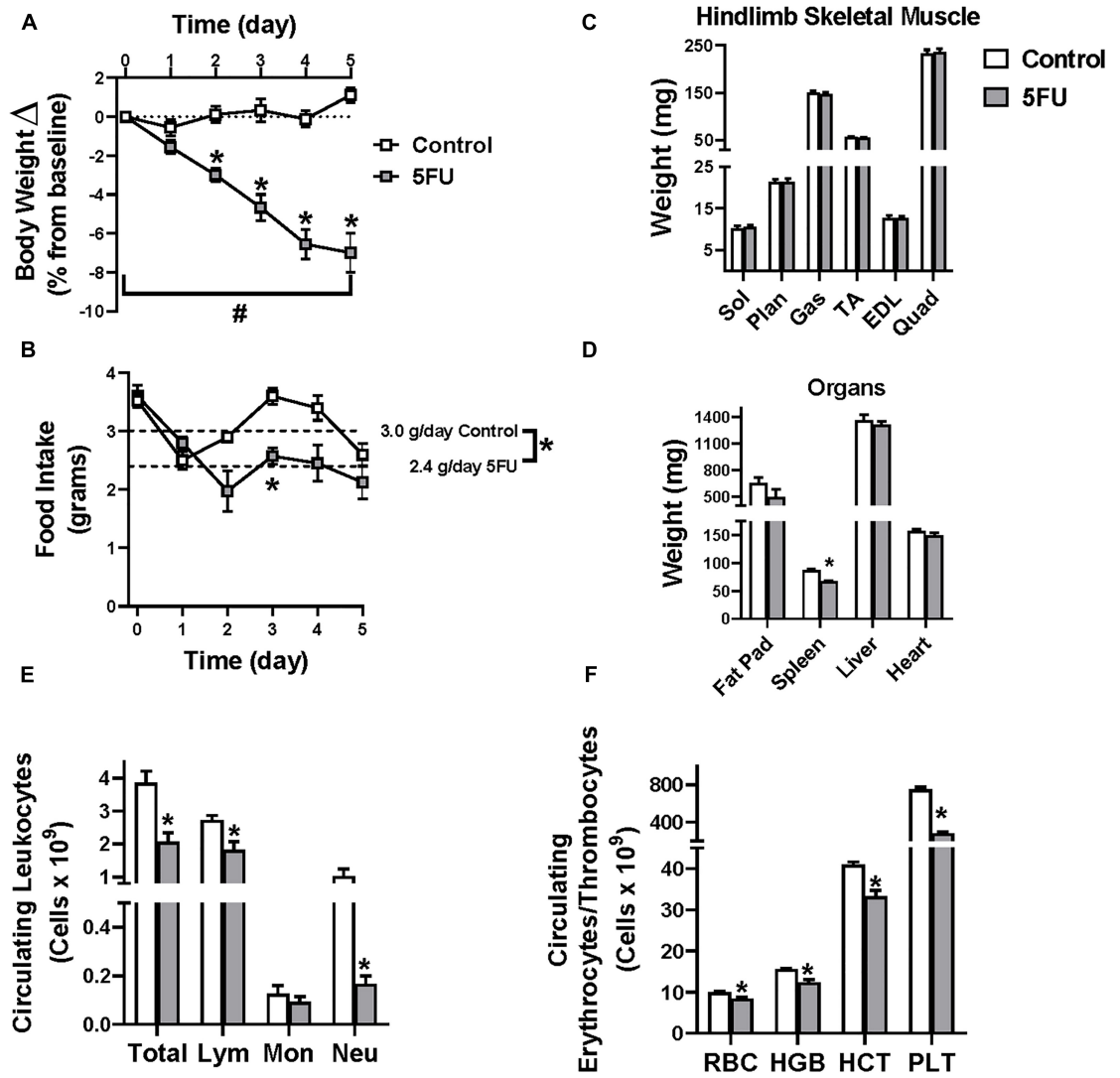

FIGURE 1 | Animal characteristics. 5-fluorouracil (5FU) was solubilized in phosphate buffered saline at 3.5 mg/mL and administered to the mice at 35 mg/kg via intraperitoneal injection once daily for 5 days. **(A)** Relative body weight change shown as the % change from day 0 throughout the duration of the study. **(B)** Daily food intake in grams throughout the duration of the study. Dotted line illustrates the average daily food intake in grams (g) per day over the course of the 5 days of treatment. **(C)** Select hindlimb muscle weights given in milligrams (mg) after 5 days of 5FU. **(D)** Select organ weights in mg after 5 days of 5FU. **(E)** Circulating leukocytes given as # of cells $\times$ 10^9/L after 5 days of 5FU. **(F)** Circulating erythrocytes and thrombocytes given as # of cells $\times$ 10^9/L after 5 days of 5FU. Sol, soleus; Plan, plantaris; Gas, gastrocnemius; TA, tibialis anterior; EDL, extensor digitorum longus; Quad, quadriceps; Lym, lymphocytes; Mon, monocytes; Neu, neutrophils; RBC, red blood cells; HBG, hemoglobin; HCT, hematocrit; PLT, platelets. Significance was set at $p < 0.05$. *Significantly different from Control using a student's *t*-test. #Significantly different from Day 0 using a repeated measures Two-way ANOVA.

muscle immune populations is essential for the development of effective treatment strategies.

Skeletal muscle immune cell depletion has been demonstrated to delay recovery and disrupt extracellular matrix remodeling leading to fibrosis, weakness, and metabolic homeostatic imbalance (Farini et al., 2007; Liu et al., 2017). While results pertaining to intrinsic skeletal muscle inflammatory signaling with several chemotherapies are equivocal, leukopenia has been well established (Kvinnsland, 1999; Yamanaka et al., 2007; Shitara et al., 2009; Baechler et al., 2010; Han et al., 2012; Abraham et al., 2015). The overall purpose of the current study was to investigate the acute effects of 5FU on resident and infiltrating skeletal muscle monocytes and inflammatory mediators. We hypothesized that an acute dosing regimen of 5FU would deplete circulating and skeletal muscle monocytes and reduce associated inflammatory cytokines consistent with systemic leukopenia. Our results demonstrate that 1 cycle of 5FU was sufficient to induce significant body weight loss and leukopenia associated with a loss of total skeletal muscle immune cells and a reduction in select inflammatory mediators. Additionally, we show 5FU induced bone marrow cell cycle arrest which is likely to contribute to the observed loss of infiltrating skeletal muscle monocytes.

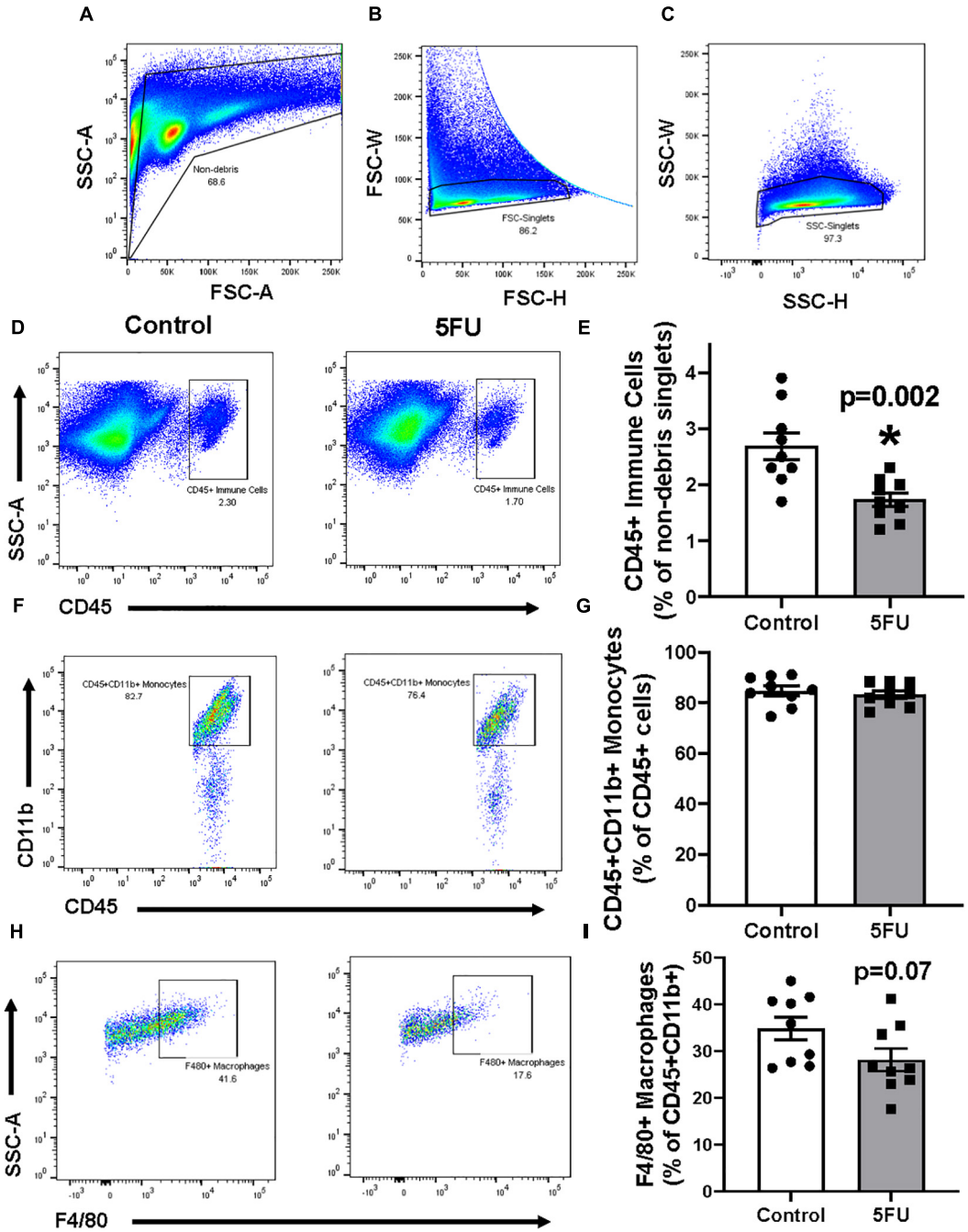

FIGURE 2 | The effects of 5FU on skeletal muscle immune cells. **(A)** Cells were gated for non-debris (SSC-A × FSC-A), **(B)** FSC singlets (FSC-W × FSC-H), **(C)** and SSC singlets (SSC-W × SSC-H; A-right). **(D)** Non-debris singlet cells were then gated for total immune cells with CD45+. **(E)** CD45+ cells were quantified and shown in the bar graph as the relative % of non-debris singlets. **(F)** CD45+ cells were gated for monocytes with CD11b+. **(G)** CD45+CD11b+ cells were quantified and shown in the bar graph as the relative % of CD45+ cells. **(H)** CD45+CD11b+ were then gated for macrophages with F4/80. **(I)** F4/80+ cells were quantified and shown in the bar graph as the relative % of CD45+CD11b+ cells. Significance was set at $p < 0.05$. *Significantly different from Control using a student's *t*-test.

MATERIALS AND METHODS

Animals

Eighteen male C57BL/6 mice were purchased from Jackson Laboratories at 4 weeks of age and housed in the Department of Laboratory Animal Resources at the University of South Carolina. Mice were either group housed (n = 12) or singly housed to measure food intake (n = 6) and kept on a 12:12-h light-dark cycle. Animals were placed on a purified AIN-76A (Bio-Serv, Frenchtown, NJ, United States; catalog#:F1515)

TABLE 1 | Skeletal muscle immune cell population.

		Total	Non-debris	FSC Singlet	SSC Singlet	CD45+	CD45+ CD11b+	CD45+ CD11b+ F4/80+
Control	Mean	500000	357171	300554	293303	8313	6986	2509
	SEM	0	(9278)	(12211)	(12898)	(1112)	(1092)	(531)
5FU	Mean	500000	329763	265158	257197	4412*	3701*	1084*
	SEM	0	(12996)	(15057)	(15433)	(397)	(384)	(182)
p-value			0.207	0.180	0.171	0.005	0.009	0.013

*Values are means ± SEM. Total number of cells counted. Absolute number of non-debris cells from the total number of cells. Absolute number of forward scatter (FSC) single cells from the non-debris cells. Absolute number of side scatter (SSC) single cells from the FSC single cells. Absolute number of CD45+ cells from the SSC single cells. Absolute number of CD45+CD11b+ cells from SSC single cells. Absolute number of CD45+CD11b+F4/80+ cells from SSC single cells. Significance was set at $p < 0.05$. *Significantly different from Control using a student's t-test.*

diet for 5 weeks prior to any experimental procedures. Body weights were measured weekly, and animals were monitored for signs of distress. Animals were given food and water *ad libitum* throughout the duration of the study. All animals were fasted 5 h prior to tissue collection. Mice were anesthetized with isoflurane and hindlimb muscles, select organs, and both femurs were carefully dissected, weighed, and either snap frozen in liquid nitrogen or placed in the appropriate buffers for flow cytometry analysis. All animal experiments were approved by the University of South Carolina's Institutional Animal Care and Use Committee.

Experimental Design

At 14 weeks of age mice were randomized into two groups, Control (n = 9) and 5FU (n = 9). 5FU was solubilized in PBS at 3.5 mg/mL and administered to the mice at 35 mg/kg i.p. once daily for 5 days. This dosing regimen has been previously shown to be comparable to clinical doses and recapitulates 1 cycle of chemotherapy (Phillips et al., 2018; Sougiannis et al., 2019). Control mice received a PBS injection. Tissue was collected and the animals were euthanized 24 h following the final injection.

Blood Analysis

Blood was collected at euthanasia via the inferior vena cava, placed in an EDTA coated vacutainer (VWR, Suwanee, GA, United States; catalog#:454428) and stored briefly on ice until analysis. A complete blood count was performed using the VetScan HMT (Abaxis, Union City, CA, United States) for determination of white blood cells (WBCs), lymphocytes (LYM), monocytes (MON), neutrophils (NEU), red blood cells (RBCs), Hemoglobin (HGB), Hematocrit (HCT), and platelets (PLT).

Flow Cytometry

Both quadriceps were excised, minced in Dulbecco's Modified Eagle Medium (DMEM), and cells were extracted using the skeletal muscle dissociation kit (Miltenyi Biotec, Auburn, CA, United States; cat#; 130-098-305) following the manufacture's instruction. Both quadriceps were pooled to obtain a sufficient number of cells for each analysis without pooling animals (n = 9/group). Skeletal muscle cells were suspended in flow buffer (0.5% BSA, 2 mM EDTA, PBS). Following hindlimb muscle excision, both femurs (n = 5/group) were cleaned and placed in ice cold PBS. The epiphysis of the femurs was removed, and the bone marrow was flushed with PBS using a 26G syringe. Cells were then passed through a 70-μm filter and suspended in flow buffer (2% FBS-PBS). Red blood cell lysis was performed with 20 second hypotonic solution (0.2% NaCl) treatment followed by hypertonic (1.6% NaCl) cessation. This method has been shown to reduce disturbances to cell surface markers compared to alternative RBC lysis buffers (Swamydas and Lionakis, 2013). Both skeletal muscle and bone marrow cells were blocked with Fc-block against CD16 and CD32 in their respective flow buffers. Cells were then incubated with fluorescently labeled antibodies against CD45 (PE/CY7), CD11b (APC), Ly6c (PerCP/Cy5.5), F4/80 (FITC), CD11c (APC/Cy7), and CD206 (PE). Cells were measured using a FACS Aria II and analyzed using FlowJo V10.6.2 (BD Biosciences, Ashland, OR, United States). Prior to cellular analysis, all colors were compensated using Invitrogen UltraComp eBeads^{TM} Compensation Beads (Life technologies, Carlsbad CA, United States). A total of 5×10^5 skeletal muscle cells and 3×10^5 bone marrow cells were analyzed.

RNA Isolation and RT-PCR

RNA isolation, cDNA synthesis, and real-time PCR were performed as previously described (Sougiannis et al., 2019) using reagents from Applied Biosystems (Foster City, CA, United States). Briefly, RNA was extracted from the gastrocnemius using the TRIzol/isopropanol/chloroform procedure (Life Technologies, GIBCO-BRL, Carlsbad, CA, United States). RNA sample quality and quantities were verified using a Nanodrop One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States) and determined to be of good quality based on A260/A280 values (>1.8) prior to cDNA synthesis using High capacity Reverse Transcriptase kit (Applied Biosystems, Foster City, CA, United States). Probes for MCP-1, IL-6, IL-1β, IL-10, TNF-α, IFNγ, CD11c, CD206, F4/80, and CD68 as well as housekeeping genes Hmbs, B2M, TBP, H2afv, and 18s were purchased from Applied Biosystems (Foster City, CA, United States). Quantitative RT-PCR analysis was carried out as per the manufacturer's instructions (Applied Biosystems, Foster City, CA, United States) using Taq-Man Gene Expression Assays on a Qiagen Rotor-Gene Q. Data were normalized to vehicle treated controls and compared to five reference targets (Hmbs, B2M, TBP, H2afv, and 18s), which were evaluated for expression stability using GeNorm (St-Pierre et al., 2017).

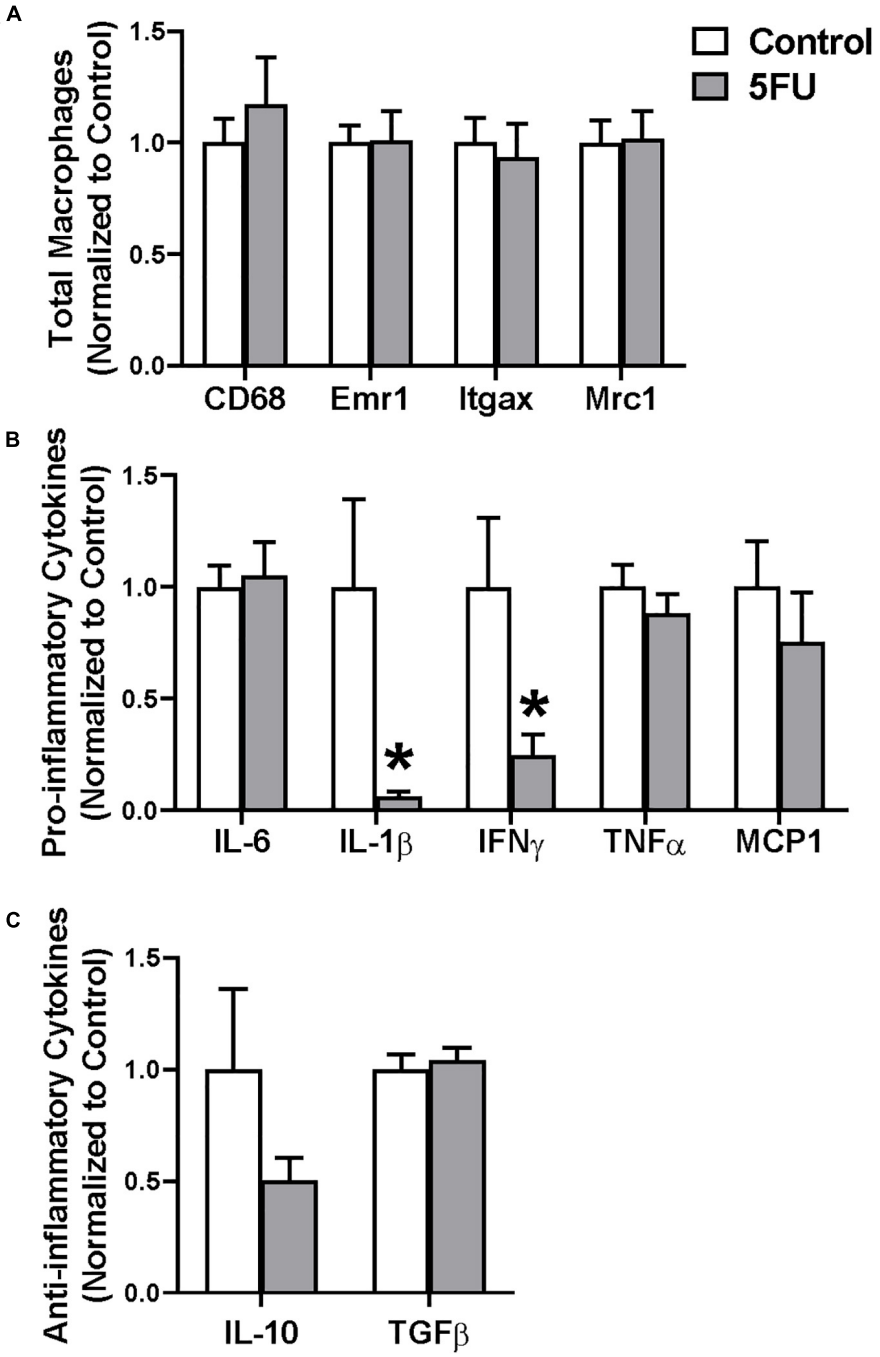

FIGURE 3 | The effects of 5FU on skeletal muscle macrophage gene expression. **(A)** Relative gene expression of total macrophage genes, CD68 and Emr1 (F4/80), M1-like macrophage gene, Itgax (CD11c), and M2-like macrophage gene, Mrc1 (CD206). **(B)** Relative gene expression of pro-inflammatory genes, Interleukin (IL) 6, IL-1β, Interferon (IFN) γ, Tumor necrosis factor (TNF) α, and monocyte chemoattractant protein (MCP) 1. **(C)** Relative gene expression of anti-inflammatory genes IL-10 and transforming growth factor (TGF) β. Significance was set at $p < 0.05$. *Significantly different from Control using a student's *t*-test.

Statistics

Values are presented as means ± standard error of the mean (SEM). Student *t*-tests were performed to determine the differences between 5FU and Control for all endpoint measurements. A repeated measures two-way ANOVA was used to determine a difference in body weight change and food intake (treatment × time). *Post hoc* analysis were performed with student Newman-Keuls methods. A Bartlett's test was used to determine significantly different standard deviations. Significance was set at $p \leq 0.05$.

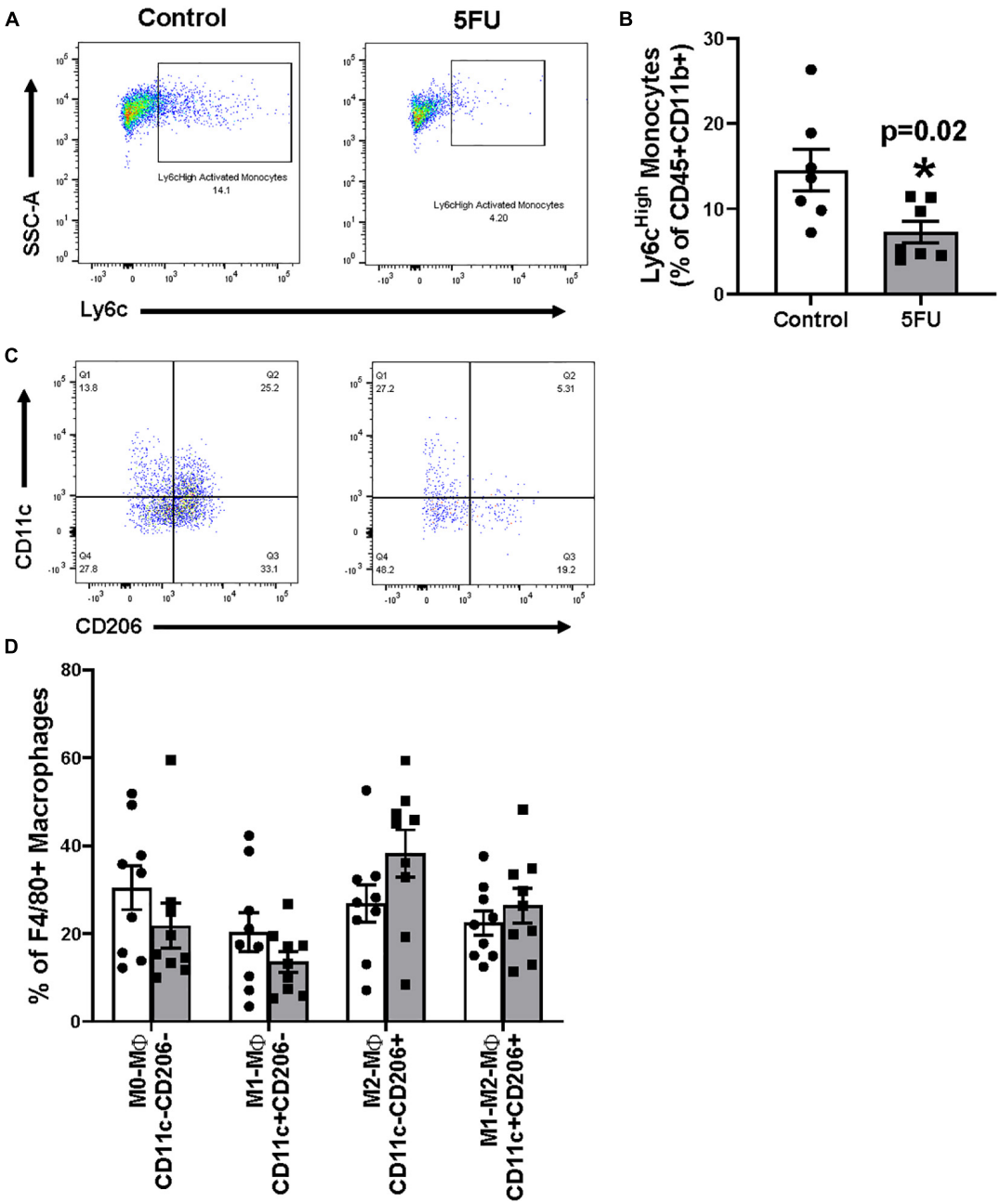

FIGURE 4 | The effects of 5FU on infiltrating skeletal muscle monocytes and macrophages. **(A)** CD11b+ monocytes were gated for their activation status using Ly6C. Cells were considered either resident (Ly6cLow) or activated/infiltrating (Ly6cHigh). **(B)** Ly6cHigh monocytes were quantified and shown in the bar graph as relative % of CD45+CD11b+ cells. **(C)** F4/80+ macrophages were gated analyzed for their polarization status using CD11c and CD206. **(D)** CD11c-CD206- cells were considered M0-like macrophages, CD11c+CD206- cells were considered M1-like macrophages, CD11c-CD206+ cells were considered M2-like macrophages, and CD11c+CD206+ cells were considered M1-M2-like transitional macrophages and graphed as the relative % of F480+ macrophages. Significance was set at $p < 0.05$.*Significantly different from control (*t*-test).

RESULTS

Animal Characteristics

Body weights were monitored daily during the treatment period and shown as a relative change (%) from Day 0. 5FU treated mice exhibited body weight loss between day 0 and Day 5 (effect of time; $p < 0.0001$) and had reduced % body weight between days 2 and 5 (2–8%) compared to controls ($p < 0.0001$) (**Figure 1A**). 5FU reduced the overall average daily food intake (g/day) by 20.5% ($p = 0.006$) compared to controls (**Figure 1B**). Despite the reductions in body weight and food intake, there were no observed differences between 5FU and controls in several

TABLE 2 | Skeletal muscle activated monocyte population.

		CD45+ CD11b+									
								F4/80+			
		Ly6c^{High}	Ly6c^{Low}	F480- Ly6c^{High}	F480+ Ly6c^{High}	F480+ Ly6c^{Low}	F480- Ly6c^{Low}	CD206- CD11c+	CD206+ CD11c+	CD206+ CD11c-	CD206- CD11c-
Control	Mean	1161	5824	685	475	1806	4018	852	385	646	625
	SEM	(398)	(701)	(230)	(168)	(337)	(387)	(222)	(131)	(150)	(132)
5FU	Mean	313*	3576*	119*	193	842*	2733*	250*	140*	423	269*
	SEM	(87)	(389)	(28)	(59)	(144)	(276)	(86)	(25)	(100)	(48)
p-value		0.060	0.016	0.031	0.141	0.022	0.019	0.030	0.015	0.248	0.044

*Values are means ± SEM. Absolute number of Ly6c^{High} cells from the CD45+CD11b+ single cells. Absolute number of Ly6c^{Low} cells from the CD45+CD11b+ single cells. Absolute number of F4/80-Ly6c^{High} cells from the CD45+CD11b+ single cells. Absolute number of F4/80+Ly6c^{High} cells from the CD45+CD11b+ single cells. Absolute number of F4/80+Ly6c^{Low} cells from the CD45+CD11b+ single cells. Absolute number of F4/80-Ly6c^{Low} cells from the CD45+CD11b+ single cells. Absolute number of CD206-CD11c+ cells from the CD45+CD11b+F4/80+ single cells. Absolute number of CD206+CD11c+ cells from the CD45+CD11b+F4/80+ single cells. Absolute number of CD206+CD11c- cells from the CD45+CD11b+F4/80+ single cells. Absolute number of CD206-CD11c- cells from the CD45+CD11b+F4/80+ single cells. Significance was set at $p < 0.05$. *Significantly different from Control using a student's t-test.*

hindlimb muscle weights, soleus ($p = 0.35$), plantaris ($p = 0.96$), gastrocnemius ($p = 0.57$), extensor digitorum longus ($p = 0.99$), tibialis anterior ($p = 0.50$), and quadriceps ($p = 0.82$) (**Figure 1C**). Spleen weight was decreased 22.6% ($p < 0.0001$) with 5FU (**Figure 1D**) which is further supported by a 46.4% ($p = 0.001$) decrease in circulating leukocytes (**Figure 1E**). More specifically, circulating lymphocytes and neutrophils were reduced with 5FU by 33.3% ($p = 0.006$) and 83.5% ($p = 0.002$), respectively, with no significant change in circulating monocytes (26.5% reduction, $p = 0.43$) (**Figure 1E**). 5FU reduced circulating red blood cells (RBC) by 16.2% ($p = 0.002$) with 20.8% ($p = 0.0003$) and 18.8% ($p = 0.0002$) reductions in hemoglobin (HGB) and hematocrit (HCT), respectively (**Figure 1F**). Additionally, 5FU decreased platelets (PLT) by 62.6% ($p < 0.0001$; **Figure 1F**).

The Effect of 5FU on Skeletal Muscle Monocytes

Cells isolated from the quadriceps underwent the following gating procedures, which was previously described (Reidy et al., 2019). Cells were first gated for "non-debris" by plotting SSC-A × FSC-A (**Figure 2A**). Cells were then gated for single cells by plotting SSC-W × SSC-H (**Figure 2B**) and then FSC-W × FSC-H (**Figure 2C**). Immune cells were then gated from "non-debris," "SSC singlets," and "FSC singlets" by plotting SSC-A by CD45. CD45+ cells were considered all immune cells and were quantified as a % of singlets (**Figure 2D**) and total number of immune cells (**Table 1**). 5FU treatment resulted in a 35.5% decrease ($p = 0.003$) in the relative quantity of CD45+ immune cells (**Figure 2E**), and a 46.9% decrease in total CD45+ immune cells (**Table 1**). CD45+ immune cells were further gated with CD11b and CD45+CD11b+ cells were classified as monocytes and were quantified as a % of CD45+ cells (**Figure 2F**) and total number of monocytes (**Table 1**). The relative abundance of monocytes within CD45+ cells was not significantly different (1.4%, $p = 0.57$) with 5FU treatment (**Figure 2G**); however, total monocytes were reduced by 47.0% with 5FU (**Table 1**). CD45+CD11b+ cells were further gated with F4/80 and CD45+CD11b+F4/80+ cells were classified as macrophages and were quantified as a % of CD45+CD11b cells (**Figure 2H**) and total number of macrophages (**Table 1**). 5FU decreased the relative abundance of macrophages by 19.2% within CD45+CD11b+ monocytes; however, this did not reach statistical significance ($p = 0.07$; **Figure 2I**). 5FU reduced total macrophage count by 56.8% (**Table 1**). Last, there was a strong correlation between circulating leukocytes and skeletal muscle CD45+ immune cells ($R = 0.75$; $p = 0.003$), CD11b+ monocytes ($R = 0.69$; $p = 0.002$), F4/80+ macrophages ($R = 0.67$; $p = 0.002$), and Ly6c^{High} infiltrating monocytes ($R = 0.67$; $p = 0.008$) in all mice.

Skeletal Muscle Inflammatory Gene Expression

RNA was extracted from the gastrocnemius which shares a similar myofibrillar myosin heavy chain isoform expression as the quadriceps and is similarly a prime mover. There was no difference in expression of total macrophage markers CD68 or Emr1 (F4/80) with 5FU treatment (**Figure 3A**). Additionally, there was no difference in M1-like macrophage gene Itgax (CD11c) or M2-like macrophage gene Mrc1 (CD206) with 5FU (**Figure 3A**). 5FU reduced pro-inflammatory cytokines, IL-1β and IFNγ, 95% ($p = 0.009$) and 75% ($p = 0.01$), respectively, while IL-6, TNFα, and MCP-1 were not changed (**Figure 3B**). There were no differences in anti-inflammatory cytokines IL-10 and TGFβ with 5FU (**Figure 3C**).

The Effect of 5FU on Resident and Infiltrating Skeletal Muscle Monocytes and Macrophages

Given the decrease in pro-inflammatory cytokines IL-1β and IFNγ, we sought to understand the phenotype of skeletal muscle monocytes. Similar to **Figure 2**, cell singlets were gated for CD45+CD11b+ followed by Ly6C to understand the effects of 5FU on infiltrating monocytes. Ly6c^{High} cells were classified as infiltrating monocytes and were quantified as a % of CD45+CD11b+ cells (**Figure 4A**) and total number of activated

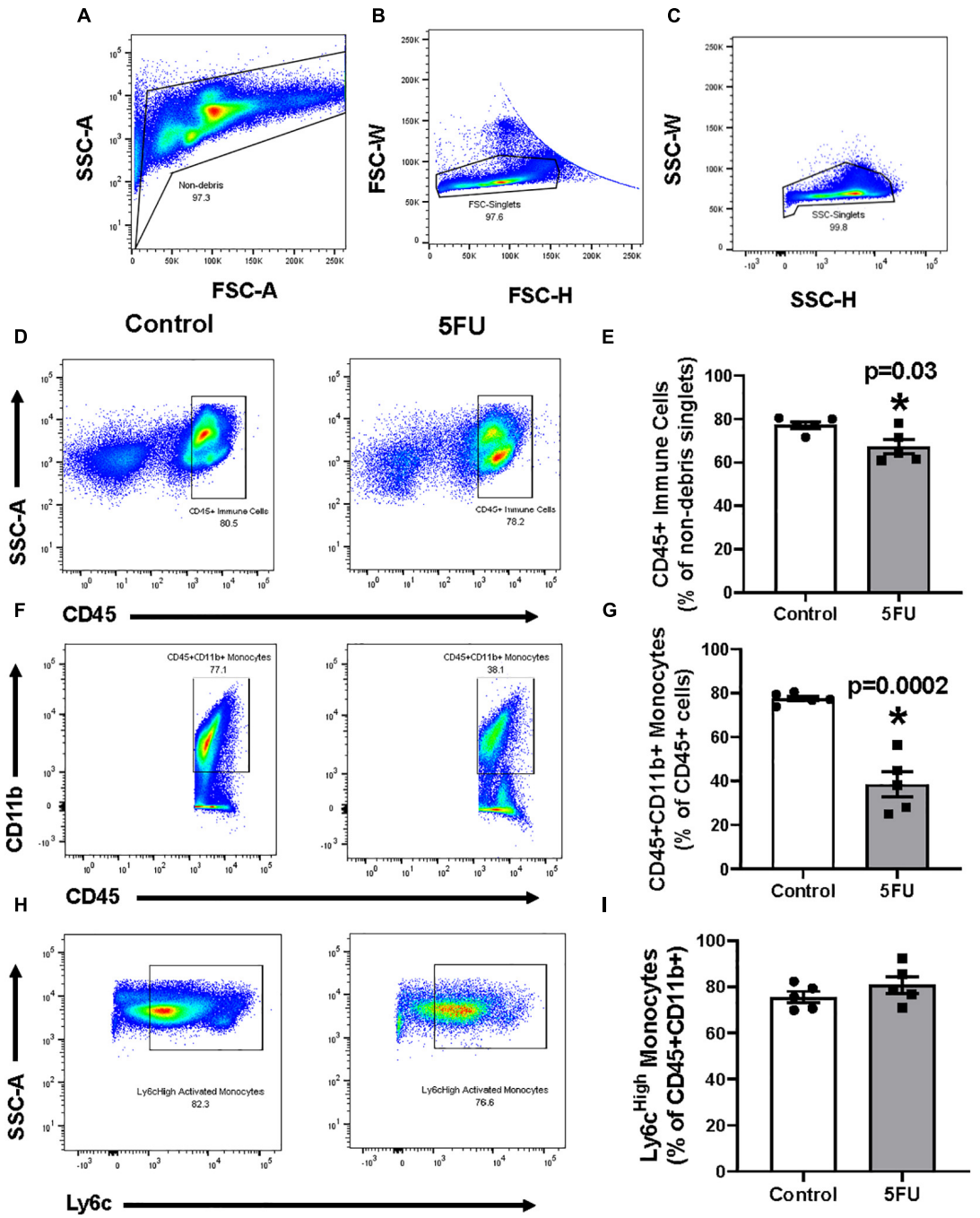

FIGURE 5 | The effects of 5FU on bone marrow immune cells. **(A)** Cells were gated for non-debris (SSC-A × FSC-A), **(B)** FSC singlets (FSC-W × FSC-H), **(C)** and SSC singlets (SSC-W × SSC-H; A-right). **(D)** Non-debris singlet cells were then gated for total immune cells with CD45+. **(E)** CD45+ cells were quantified and shown in the bar graph as the relative % of non-debris singlets. **(F)** CD45+ cells were gated for monocytes with CD11b+. **(G)** CD45+CD11b+ cells were quantified and shown in the bar graph as the relative % of CD45+ cells. **(H)** CD11b+ monocytes were gated for their activation status using Ly6C. Cells were considered either resident (Ly6cLow) or activated/infiltrating (Ly6cHigh). **(I)** Ly6cHigh monocytes were quantified and shown in the bar graph as relative % of CD45+CD11b+ cells. Significance was set at $p < 0.05$. *Significantly different from Control using a student's *t*-test.

monocytes (**Table 2**). 5FU decreased the relative abundance of Ly6c^{High} infiltrating monocytes by 49.9% ($p = 0.02$) within CD45+CD11b+ monocytes (**Figure 4B**). Also, total Ly6c^{High} infiltrating monocytes were reduced by 73.0% with 5FU, but this did not achieve statistical significance ($p = 0.06$; **Table 2**). Total Ly6c^{Low}, resident monocytes, were reduced by 38.6% with 5FU (**Table 2**). Given that the total number of macrophages were reduced with 5FU (**Table 1**), we examined if there were

TABLE 3 | Bone marrow immune cell population.

		Total	Non-debris	FSC Singlet	SSC Singlet	CD45+	CD45+ CD11b+	CD45+ CD11b+ Ly6c^{High}
Control	Mean	500000	483227	465831	464058	304878	233494	160170
	SEM	0	(1160)	(2145)	(2295)	(50575)	(44979)	(40441)
5FU	Mean	500000	409499*	401998*	400736*	246100	91678*	74938*
	SEM	0	(8611)	(8845)	(8948)	(26514)	(19190)	(16942)
p-value			0.00001	0.00003	0.00003	0.096	0.001	0.010

*Values are means ± SEM. Total number of cells counted. Absolute number of non-debris cells from the total number of cells. Absolute number of forward scatter (FSC) single cells from the non-debris cells. Absolute number of side scatter (SSC) single cells from the FSC single cells. Absolute number of CD45+ cells from the SSC single cells. Absolute number of CD45+CD11b+ cells from SSC single cells. Absolute number of CD45+CD11b+Ly6c^{High} cells from SSC single cells. Significance was set at $p < 0.05$. *Significantly different from Control using a student's t-test.*

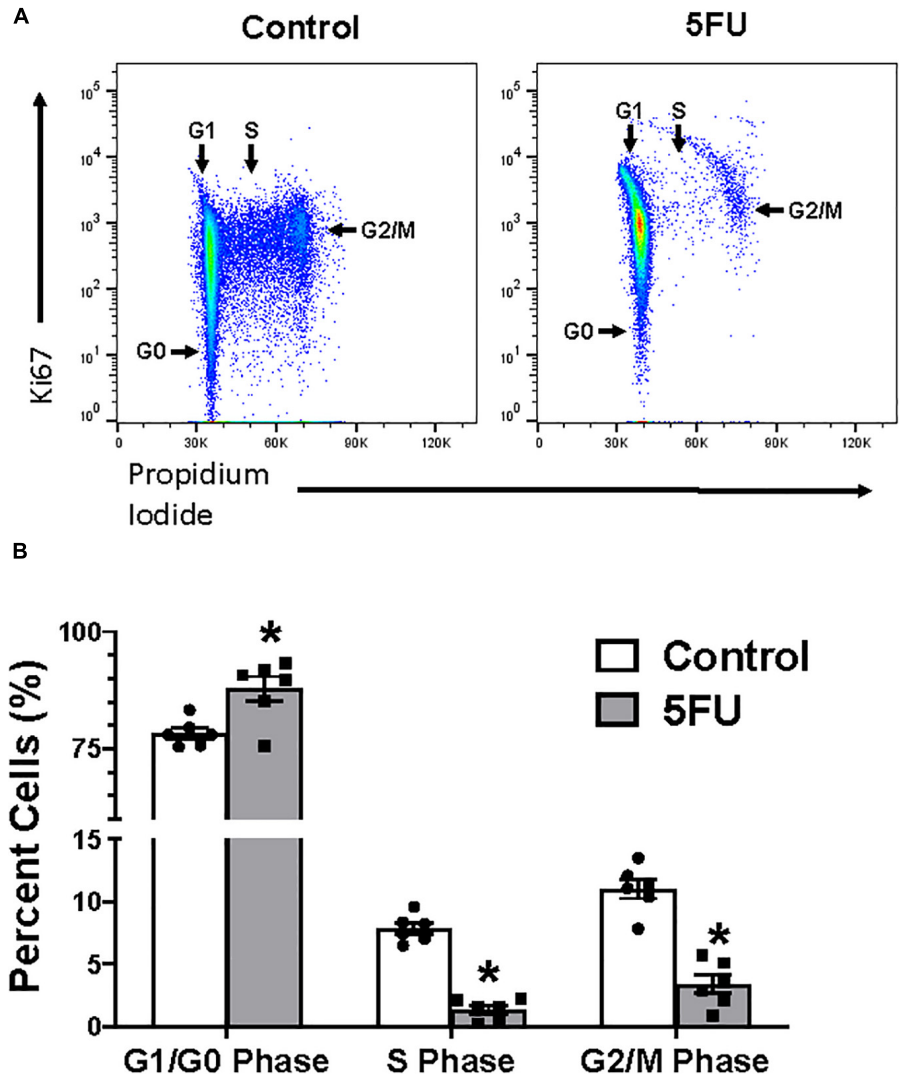

FIGURE 6 | The effects of 5FU on bone marrow cell cycle. **(A)** Cells were fixed and stained with Ki67 and propidium iodide (PI). **(B)** Cells in the G1/G0, S, and G2/M phases were quantified and shown in the bar graph as the relative % of total cells. Significance was set at $p < 0.05$.*Significantly different from control (*t*-test).

changes in macrophage phenotype by measuring CD11c (M1-like) and CD206 (M2-like) from parent CD45+CD11b+F4/80+ macrophages (**Figure 4C**). There were no observed changes in the relative abundance of M1-like ($p = 0.20$), M2-like ($p = 0.12$), M1-M2 transitional ($p = 0.42$), or M0 ($p = 0.24$) macrophages with 5FU treatment (**Figure 4D**); however, 5FU decreased total number of M1-like (CD11c+CD206-) by 70.7%, M1-M2 transition (CD11c+CD206+) by 63.6%, and M0

(CD11c-CD206-) macrophages by 57.0% (**Table 2**). The total number of M2-like macrophages (CD11c-CD206+) cells were not significantly different between groups (**Table 2**).

The Effects of 5FU on the Bone Marrow

In order to further understand the effects of 5FU on circulating and infiltrating monocytes, we examined 5FU's impact on bone marrow cells (**Figure 5**). Bone marrow isolates were obtained from both femurs of 5 mice/group. Cell gating of bone marrow cells was performed as described for data in **Figures 2**, **5A–D**. 5FU decreased the relative abundance of CD45+ immune cells by 12.9% ($p = 0.03$; **Figure 5E**) and total CD45+ immune cells by 19.3% (**Table 3**), but the reduction in total CD45+ immune cells did not reach statistical significance ($p = 0.096$). CD45+ cells were further gated with CD11b and CD45+CD11b+ cells were considered monocytes (**Figure 5F**) and were quantified as a % of CD45+ cells (**Figure 5G**) and total number of monocytes (**Table 3**). 5FU treatment reduced the relative abundance of bone marrow monocytes by 50.3% ($p = 0.0002$) within total CD45+ immune cells (**Figure 5G**) and reduced total monocytes by 60.7%. CD45+CD11b+ cells were further gated with Ly6C and CD45+CD11b+Ly6C^{High} cells were considered activated monocytes (**Figure 5H**) and were quantified as a % of CD45+CD11b+ cells (**Figure 5G**) and total number of activated monocytes (**Table 3**). 5FU treatment had no apparent effect on the relative abundance of bone marrow activated monocytes within total CD45+CD11b+ monocytes (**Figure 5I**); however, the total number of activated monocytes was reduced by 53.2% (**Table 3**). Additionally, 5FU induced cell cycle arrest in the bone marrow (**Figure 6**). 5FU increased the relative abundance of cells in the G1/G0 cell cycle phase by 12.0% ($p = 0.009$) and decreased S and G2/M cell cycle phases by 82.4% ($p < 0.0001$) and 69.1% ($p < 0.0001$), respectively (**Figure 6B**).

DISCUSSION

5 fluorouracil has been the first-choice chemotherapy drug for several cancer types; however, its efficacy is diminished by patient acquired resistance and pervasive side effects contributing to reduced life quality and poor treatment outcomes (van Kuilenburg and Maring, 2013; Giuliani and Bonetti, 2016; Lee et al., 2016; McQuade et al., 2017). Given 5FU's deleterious effects on circulating leukocytes, the purpose of our study was to investigate the acute effects of 5FU on resident and infiltrating skeletal muscle monocytes and inflammatory mediators. Our results extend previous studies to identify that 1 cycle of a clinically translatable dose of 5FU significantly reduced CD45+ immune cells and infiltrating/activated CD11b+Ly6C^{High} monocytes in skeletal muscle that was associated with a decrease in select skeletal muscle inflammatory mediators. Additionally, the reduction in skeletal muscle was accompanied by a reduction in bone marrow monocytes and an increase in cell cycle arrest. These results identify novel off-target effects of 5FU on skeletal muscle and the skeletal muscle microenvironment independent of muscle mass regulation.

Our understanding of chemotherapy-induced body weight and function loss, termed cachexia, has improved over the last decade (Gilliam et al., 2009; Barreto et al., 2016b; Morton et al., 2019). Our investigation of the acute (1 cycle) effects of 5FU demonstrated that 5FU induced clinically relevant body weight loss (>5%) (Evans et al., 2008), which was accompanied with signs of anorexia, but not skeletal muscle mass loss. Others have demonstrated that 5 weeks of 5FU combination therapy, Folfiri (leucovorin, 5FU, Irinotecan), decreased body weight and lean mass over time, with corresponding reductions in several hindlimb weights (Barreto et al., 2016b). Interestingly, mice given 5 weeks of Folfox (leucovorin, 5FU, oxaliplatin) rather than Folfiri maintained body weight and lean mass, and only showed reduced quadriceps weight. Additionally, similar to the doxorubicin effects on skeletal muscle (Gilliam et al., 2009; Tarpey et al., 2019), only Folfiri reduced skeletal muscle specific force (strength per muscle unit area) – which may occur through several mechanisms including fibrosis (Barreto et al., 2016b). Together this suggests that significant muscle mass loss only occurs after sustained 5FU treatment (Barreto et al., 2016b, 2017), given that 1 week of 5FU was unable to reduce hindlimb muscle weight. We then hypothesize that it is likely that anorexia and dehydration contribute to the observed body weight loss as these are commonly reported with 5FU (Liaw et al., 1999; Yi et al., 2016). Additionally, the potential for body weight loss and anorexia alone to contribute to the observed immune disruptions cannot be ruled out. Unfortunately, however, a weight loss only group was not included to test this hypothesis therefore limiting our interpretations. Nonetheless, our results provide evidence to suggest that 5FU-induced anemia and leukopenia are likely to contribute to the observed functional pathologies that occur with 5FU treatment.

The role of immune cells, particularly macrophages, in skeletal muscle regeneration, repair, and remodeling has been well characterized (Tidball, 2017); however, chemotherapy's effects on these processes is not well known. Following skeletal muscle insult (e.g., damage, ischemia, exercise), there is an initial influx of neutrophils which in turn recruit naïve monocytes primarily through the release of MCP-1 (Deshmane et al., 2009; Tidball, 2017). We have previously shown that 5FU induced circulating MCP-1 after 14 days of treatment, which was associated with reduced voluntary physical activity (Mahoney et al., 2013). The monocytes recruited by MCP-1 are primarily recruited as CD11b+Ly6C^{High} monocytes which can either remain as such or differentiate and polarize to a pro-inflammatory M1-like F4/80+CD11c+CD206- macrophage (Tidball, 2005; Guilliams et al., 2014; Yang et al., 2014). Following an acute 5FU regime (1 week) we document a reduction in total and relative Ly6C^{High} monocytes as well as total M1-like F4/80+CD11c+CD206- macrophages in skeletal muscle despite no changes in skeletal muscle pro-inflammatory MCP-1, IL-6, and TNFα levels. However, we did observe decreased expression of pro-inflammatory genes associated with M1-like macrophages, IL-1β and IFNγ, but on the other hand did not observe corresponding changes to total M1-like macrophage cell surface marker, Itgax, more commonly known as CD11c (Jablonski et al., 2015). These discrepancies between the flow

cytometry and gene transcription require additional work and thus, interpretations should be taken with caution; however, flow cytometry remains the gold standard for the assessment of immune cells, and it appears evident that 5FU has deleterious effects on the pro-inflammatory monocytes and macrophages. A loss of pro-inflammatory or phagocytic M1-like macrophages could negatively impact skeletal muscle remodeling and repair (Tidball, 2005). Chemotherapeutic doxorubicin has been shown to blunt the pro-inflammatory response following exercise which mitigated the muscle's response to exercise (Huang et al., 2017). Furthermore, while repeated muscular contractions were able to improve muscle mass in cancer patients undergoing treatment, patients did not obtain the functional and metabolic improvements that have been previously seen with exercise (Guigni et al., 2019; Toth et al., 2020). While chemotherapeutics 5FU and doxorubicin mechanisms of action differ, we can still glean potential mechanisms and clinical manifestations. To the best of our knowledge, we are the first to document that 5FU disrupts skeletal muscle's pro-inflammatory immune cell environment. It is also important to note that these cell surface markers and the M1/M2 dichotomous classification of macrophages does not properly reflect the true diversity and nature of resident/infiltrating macrophages and should again be interpreted cautiously (Davies et al., 2013; Guilliams et al., 2014; Martinez and Gordon, 2014; Tidball, 2017).

Tissue resident macrophages are classically CD206+ anti-inflammatory, pro-fibrotic surveying macrophages (Gordon and Taylor, 2005; Murray and Wynn, 2011; Cote et al., 2013; Guilliams et al., 2014); however, macrophages are plastic and as skeletal muscle repair progresses the infiltrated M1-like F4/80+CD11c+ macrophages can reduce the gene expression and release of pro-inflammatory mediators and become more phenotypically M2-like to promote extracellular matrix remodeling and angiogenesis (Schiaffino et al., 2017; De Santa et al., 2018; Shapouri-Moghaddam et al., 2018). Others have proposed that resident macrophages are predominantly M0 (CD11c-CD206-) which are self-maintained, proliferate, and polarize to an M1-like phenotype upon activation during the initial stages of injury repair (Cote et al., 2013; Tidball, 2017). Regardless, our results demonstrate that the relative phenotype of skeletal muscle macrophages is not changed by 5FU treatment; however, the total number of M1-like (CD11c+CD206-), M0-like (CD11c-CD206-), and M1-M2-like transitional macrophages were reduced with 5FU while M2-like macrophages appear spared from 5FU's cytotoxicity – at least following 1 week of 5FU. Additionally, anti-inflammatory IL-10, pro-fibrotic TGFβ, and M2-like macrophage cell surface marker Mrc1, commonly known as CD206, gene transcription were not changed by 5FU treatment. The potential for 5FU to target M1-like macrophages rather than M2-like, points to a pro-fibrotic skeletal muscle microenvironment. 5FU combination therapy Folfiri was shown to reduce skeletal muscle specific force (force per unit area); however, neither fibrosis nor an increase in fibrotic genes (TGF-β associated ligands) were apparent (Barreto et al., 2016b). Therefore, it is likely that these pro-fibrotic M2-like cells remain at a physiological abundance during 5FU treatment and may not be contributing to a skeletal muscle pathology directly. Interestingly, TAMs phenotypically reflect M2-like macrophages promoting immunosuppression, fibrosis, and angiogenesis, within the tumor microenvironment and have been associated with 5FU acquired resistance (Zhang et al., 2016). The potential for M2-like macrophages to be protected against 5FU requires significant attention in the cancer domain.

Chemotherapy has been shown to mitigate the inflammatory response with exercise (Huang et al., 2017; Smuder, 2019), induce leukopenia/cytopenia (Shitara et al., 2011), and disrupt cardiac macrophage infiltration (Johnson and Singla, 2018). To the best of our knowledge, this is the first study to demonstrate that chemotherapeutic 5FU has deleterious effects on immune cell abundance in otherwise healthy uninjured skeletal muscle. The absolute reduction in macrophage number rather than relative changes in abundance remains relevant given the physiological importance of the overall immune response in repair and remodeling (Summan et al., 2006; Segawa et al., 2008; Cote et al., 2013; Xiao et al., 2016; Zhao et al., 2016; Inaba et al., 2018). The mean age of cancer patients is ∼65 years and overlapping sarcopenic and cachectic factors along with chemotherapy may contribute to disrupted skeletal muscle immune regulation (Dunne et al., 2019). Disrupted skeletal muscle repair associated with changes in macrophages has been reported with aging (Reidy et al., 2019), cancer (Inaba et al., 2018), and chemotherapy (Huang et al., 2017). The effects of aging on skeletal muscle macrophages has demonstrated that reloading aged skeletal muscle had a blunted hypertrophy response associated with a lower number of M1-like macrophages at baseline and blunted M1-like macrophage infiltration (early) and M2-like macrophage transition (late) (Reidy et al., 2019). Surprisingly, while inflammation is a hallmark of cancer cachexia associated with muscle weakness and fatigue (VanderVeen et al., 2017, 2018), total macrophage number was reduced in damaged muscle of C26 tumor-bearing mice compared to a non-cachectic tumor-bearing control (Inaba et al., 2018). Additionally, macrophages were shown to regulate skeletal muscle signal transducer and activator of transcription 3 (STAT3) – downstream target of IL-6 and key regulator of skeletal muscle mitochondrial homeostasis and proteostasis (Carson and Kristen, 2010; Bonetto et al., 2011, 2012; VanderVeen et al., 2017, 2019) – during pancreatic cancer cachexia (Shukla et al., 2020). Further work is needed to understand these potentially overlapping mechanisms with cancer and chemotherapy on skeletal muscle immune cells.

Chemotherapy's effects on systemic inflammatory mediators (Lee et al., 2014; Ribeiro et al., 2016; Derman et al., 2017; Sougiannis et al., 2019) and intrinsic skeletal muscle inflammatory signaling (Kvinnsland, 1999; Yamanaka et al., 2007; Shitara et al., 2009; Baechler et al., 2010; Han et al., 2012; Abraham et al., 2015) are continuing to be unearthed; however, our study is the first to identify that 5FU-induced leukopenia extends beyond circulation to impact the skeletal muscle microenvironment. Our results indicate that 5FU's toxic effects on skeletal muscle leukocytes are not necessarily specific to monocytes shown by no change in both circulating monocyte count or relative abundance of skeletal muscle CD11b+ monocytes within the CD45+ population. This is not to say that monocytes are spared from 5FU as the total number of skeletal muscle

monocytes are reduced. Bone marrow CD11b+ monocytes and the relative abundance of infiltrating Ly6C^{High} monocytes in skeletal muscle were reduced with 5FU which is supported by the established deleterious effects of 5FU on circulating leukocytes and the hematopoietic system (Yamanaka et al., 2007; Lee et al., 2016; Sougiannis et al., 2019). In conjunction with previous studies, our results support that 5FU's toxicity is predominantly associated with pro-inflammatory mediators extending beyond the hematopoietic system to impact the skeletal muscle microenvironment. Another potential mechanism for the observed impact of 5FU on skeletal muscle immune cells is the potential for reduced proliferation of pro-inflammatory macrophages within the muscle microenvironment. Given that circulating and skeletal muscle monocytes/macrophages are not proportionally reduced it is possible that 5FU increased maturation of monocytes within the skeletal muscle as well as increased proliferation of M2-like macrophages.

CONCLUSION

Understanding chemotherapy's off-target effects will allow for improvements to treatment efficacy aimed at increasing cancer patient survival and quality of life. Our novel finding that chemotherapeutic 5FU depletes skeletal muscle immune cells and infiltrating monocytes provides insight into the skeletal muscle microenvironment that may contribute to weakness, fatigue, and treatment intolerance (Williams et al., 2018). We provide evidence to suggest that 5FU reduced circulating and skeletal muscle leukocytes through disrupting the hematopoietic system by inducing cell cycle arrest in the bone marrow. While our results are limited to 5FU's acute toxicities, future studies are needed to understand the long-term implications of this loss of immune cells and if chronic 5FU exposure exacerbates this immune dysregulation. Furthermore, additional work is needed to determine if mitigating the loss of immune cells can improve skeletal muscle function following repeated cycles of 5FU.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the University of South Carolina's Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

BV, JC, KV, DF, and EM conceived and designed the experiments. BV and AS performed the experiments. BV prepared the figures and drafted the manuscript. All authors edited, revised, and approved final version of the manuscript.

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Activation of Tripartite Motif Containing 63 Expression by Transcription Factor EB and Transcription Factor Binding to Immunoglobulin Heavy Chain Enhancer 3 Is Regulated by Protein Kinase D and Class IIa Histone Deacetylases

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Rationale: The ubiquitin–proteasome system (UPS) is responsible for skeletal muscle atrophy. We showed earlier that the transcription factor EB (TFEB) plays a role by increasing E3 ubiquitin ligase muscle really interesting new gene-finger 1(MuRF1)/*tripartite motif-containing 63* (*TRIM63*) expression. MuRF 1 ubiquitinates structural proteins and mediates their UPS-dependent degradation. We now investigated how TFEB-mediated *TRIM63* expression is regulated.

Objective: Because protein kinase D1 (PKD1), histone deacetylase 5 (HDAC5), and TFEB belong to respective families with close structural, regulatory, and functional properties, we hypothesized that these families comprise a network regulating *TRIM63* expression.

Methods and Results: We found that TFEB and transcription factor for immunoglobulin heavy-chain enhancer 3 (TFE3) activate *TRIM63* expression. The class IIa HDACs HDAC4, HDAC5, and HDAC7 inhibited this activity. Furthermore, we could map the HDAC5 and TFE3 physical interaction. PKD1, PKD2, and PKD3 reversed the inhibitory effect of all tested class IIa HDACs toward TFEB and TFE3. PKD1 mediated nuclear export of all HDACs and lifted TFEB and TFE3 repression. We also mapped the PKD2 and HDAC5 interaction. We found that the inhibitory effect of PKD1 and

PKD2 toward HDAC4, HDAC5, and HDAC7 was mediated by their phosphorylation and 14-3-3 mediated nuclear export.

Conclusion: TFEB and TFE3 activate *TRIM63* expression. Both transcription factors are controlled by HDAC4, HDAC5, HDAC7, and all PKD-family members. We propose that the multilevel PKD/HDAC/TFEB/TFE3 network tightly controls *TRIM63* expression.

Keywords: muscle atrophy, protein kinase D, HDAC = histone deacetylase, transcription factor EB, TFE3, muscle ring finger protein 1

INTRODUCTION

Muscle mass is mainly regulated by an equilibrium of protein synthesis and degradation. Both muscle atrophy and hypertrophy occur physiologically and under disease conditions (Hershko and Ciechanover, 1998; Ciechanover, 2006; Schmidt et al., 2014; Wollersheim et al., 2014). The ubiquitin–proteasome system (UPS) is the principal protein-degrading system in muscle and largely responsible for the degradation of contractile proteins (Medina et al., 1995; Wing et al., 1995; Voisin et al., 1996; Ciechanover, 2006; Witt et al., 2008). Substrate protein ubiquitination is mediated by E3 ubiquitin-ligases that are specificity assuring and rate-limiting enzymes within the UPS (Hershko and Ciechanover, 1998; Ciechanover, 2006). Polyubiquitinated target proteins are then degraded by the 26S proteasome (Ciechanover, 2006). The UPS is activated during muscle atrophy leading to declining of structural and contractile proteins, most notably myosin heavy chain (MHC) (Fielitz et al., 2007), resulting in a reduction of muscle mass and function. The E3 ligase, muscle really interesting new gene-finger 1 (MuRF1), was identified as a major atrogene and is restricted to skeletal muscle and the heart (Bodine et al., 2001a). MuRF1 was shown to mediate ubiquitination and UPS-dependent degradation of structural proteins, such as alpha-actin, troponin I, troponin T, telethonin, titin, nebulin, the nebulin-related-anchoring protein, myosin light chain 2, myotilin, and T-cap (Kedar et al., 2004; Witt et al., 2005; Polge et al., 2011, 2018). Importantly, MuRF1 is also involved in muscular energy metabolism by degradation of non-structural proteins, such as muscle-type creatine kinase, glucocorticoid modulatory element binding protein-1, and 3-hydroxyisobutyrate dehydrogenase in striated muscles (McElhinny et al., 2002; Koyama et al., 2008). Numerous studies showed that the *tripartite motif-containing 63* (*TRIM63*) gene expression, encoding MuRF1, is increased rapidly and strongly during various physiological and pathological atrophy conditions (Bodine et al., 2001a). The multitude of its target proteins, together with its strong transcriptional regulation, highlights the importance of MuRF1 in muscle homeostasis. To evaluate the transcriptional control of *TRIM63*, we performed a complementary DNA (cDNA)-expression screen to identify regulators of *TRIM63* expression and identified the basic helix-loop-helix leucine zipper (bHLH-LZ) transcription factor EB (TFEB) as a novel *TRIM63* regulator (Du Bois et al., 2015). We found that TFEB binds to specific, well-conserved, enhancer box (E-box) DNA motifs in the *TRIM63* promoter that are close to its transcription start site. We reported that TFEB activity was inhibited via interaction with the class IIa histone deacetylase (HDAC) 5. We also showed that inhibition of TFEB by HDAC5 was attenuated by the stress-dependent serine/threonine kinase protein kinase D (PKD) 1, which interacted with HDAC5 and mediated its phosphorylation and 14-3-3 chaperone mediated nuclear export. This PKD1/HDAC5/TFEB axis controlled the expression of *TRIM63* and was found to be important in angiotensin II-induced myocyte atrophy *in vitro* and muscle atrophy *in vivo* (Du Bois et al., 2015).

Recent reports, however, indicated that this pathway might not be as specific as we believed. For example, TFEB belongs to the microphthalmia/transcription factor E (MiT/TFE) family of bHLH-LZ transcription factors (TFs), which also includes TFE3, TFEC, and microphthalmia-associated transcription factor (MiTF). All MiT/TFEs recognize a unique E-box motif within the proximal promoters of lysosomal and autophagy genes (Sardiello et al., 2009; Palmieri et al., 2011; Martina et al., 2014) and regulate cellular catabolism and nutrient-dependent lysosomal response (Settembre et al., 2011; Slade and Pulinilkunnil, 2017). Importantly, TFEB and TFE3 were found to have partially redundant functions and to regulate overlapping sets of genes (Pastore et al., 2016). However, whether or not TFE3 or MiTF can activate *TRIM63* expression is not known. In addition, the class IIa HDACs (HDAC4, HDAC5, HDAC7, and HDAC9) are highly expressed in the heart and skeletal muscle, and their interaction with the myocyte enhancer factor 2 (MEF2) TF decreases the expression of MEF2 target genes (Lu et al., 2000a,b; McKinsey et al., 2000; Zhang et al., 2002). If other class IIa HDACs inhibit the activity of TFEB or TFE3 is also unclear. Finally, PKD1 belongs to a family of calmodulin-calcium-dependent serine-threonine kinases (termed PKD1, PKD2, and PKD3). These kinases are important in cell growth, differentiation, migration, and apoptosis (Rozengurt et al., 2005). The close structural relationship of the PKD family members suggested that they might phosphorylate overlapping targets. Indeed, all three family members PKD1, PKD2, and PKD3 were shown to phosphorylate HDAC5 (Huynh and McKinsey, 2006). However, any effect on TFEB or TFE3-mediated *TRIM63* expression is uncertain. Because PKD1, HDAC5, and TFEB belong to respective families with close structural, regulatory, and functional properties, we

Abbreviations: HDAC, histone deacetylase-5; MuRF, Muscle really interesting new gene (RING)-finger containing protein; MiT/TFE, Microphthalmia-associated transcription factor/transcription factor E; PKD, Protein Kinase D; TF, transcription factor; TFEB, transcription factor EB; TFE3, Transcription factor for immunoglobulin heavy-chain enhancer 3; *TRIM63*, Tripartite motif-containing 63 (mouse gene encoding MuRF1).

hypothesized that other family members could also be involved in the regulation of *TRIM63* expression.

MATERIALS AND METHODS

Cell Culture, Complementary DNA Expression Plasmids, and Luciferase Reporter Assays

Cell culture experiments of murine myoblasts (C2C12 cells, ATCC, United States) were performed as previously described (Langhans et al., 2014; Huang et al., 2017; Zhu et al., 2017). Briefly, myoblasts were cultured in the growth medium [Dulbecco's modified Eagle's medium (1 g/l glucose, Merck, Germany), 10% fetal bovine serum (Biochrom GmbH, Germany), supplemented with penicillin and streptomycin (Merck, Germany)]. For Western blot analyses, immunostaining and co-immunoprecipitation experiments, the C2C12 cells were cultivated on six-well plates, coverslips and 10 cm dishes, respectively. COS-7 cells were cultured in standard cell culture conditions using Dulbecco's modified Eagle medium (4.5 g/l glucose, L-glutamine, 10% fetal bovine serum, and penicillin–streptomycin). Cells were seeded in 24-well plates in triplicates and transfected with cDNA expression plasmids, vector control, and pGL3-*TRIM63*_Luc reporter construct, as indicated, using FuGENE6® (Promega) transfection reagent according to the manufacturer's protocol. To control transfection efficacy, 25 ng of pCMV lacZ (Clontech) was co-transfected in each sample. Cell pellets were lysed in 200 µl Luciferase Cell Culture Lysis Reagent (Promega). Fifty microliters of lysate was used for quantification of luciferase activity and β-galactosidase in a luminometer (FLUOstar Optima, BMG-Labtech). The Luciferase Assay System (Promega) was used to quantify the expression of the reporter gene constructs. Luciferase activity was normalized to fluorescence measured with the FluoReporter® lacZ/Galactosidase Quantification Kit (Invitrogen). The cDNA expression plasmids [pcDNA3.1-TFEB-C-MYC, pcDNA3.1-TFEB-N-FLAG, pcDNA3.1-PKD1-CA-N-MYC, pcDNA3.1-HDAC4-MYC, pcDNA3.1-HDAC5-MYC, pcDNA3.1-HDAC7-MYC, pcDNA3.1-C-MYC, pcDNA3.1-C-MYC, and pcDNA3.1-N-FLAG (Vega et al., 2004; Fielitz et al., 2008; Kim et al., 2008; Du Bois et al., 2015), HDAC5-deletion mutants (Song et al., 2006; Du Bois et al., 2015), and further constructs (pGL3-*TRIM63*_Luc, pGL3-*TRIM63*_mut_E-box1_Luc, pGL3-*TRIM63*_mut_E-box2_Luc, pGL3-*TRIM63*_mut_E-box3_Luc, and pCMV lacZ) (Fielitz et al., 2008; Kim et al., 2008; Du Bois et al., 2015)] were recently published. The cDNA expression plasmids for human HDAC1 (#13820) (Emiliani et al., 1998), human HDAC3 (#13819) (Emiliani et al., 1998), and human calcium/calmodulin-dependent protein kinase type IV (CamK IV, #126422) (Xie and Black, 2001) were purchased from Addgene, United States. For the generation of the cDNA expression vectors pcDNA3.1-TFE3-N-FLAG, pcDNA3.1-MiTF-N-FLAG, pcDNA3.1-PKD2-CA-N-MYC, pcDNA3.1-PKD3-CA-N-MYC, pcDNA3.1-PKD2-CA-N-FLAG, and pcDNA3.1-PKD3-CA-N-FLAG, expressing murine TFE3, MiTF, constitutive active PKD2 or PKD3 with an N-terminal FLAG tag or an N-terminal MYC tag, respectively, mouse cDNA was PCR amplified with the primers shown in Online **Table 1** using Advantage HD Polymerase (Takara) and cloned into pcDNA-3.1-N-MYC or pcDNA-3.1-N-FLAG (both Invitrogen) using the restriction enzymes indicated and T4 DNA ligase (both New England Biolabs) according to the manufacturer's protocol. All constructs were verified by sequencing. The mammalian two-hybrid assay was performed as recently published (Chang et al., 2005; Du Bois et al., 2015).

Immunofluorescence

For immunostaining, C2C12 myoblasts were cultured in 8 chamber polystyrene vessel tissue culture treated glass slides (BD Biosciences, United States) and fixed with 4% paraformaldehyde/PBS, permeabilized with 0.2% Triton-X-100/PBS, and blocked with 2% goat serum/PBS. The following primary antibodies were used: anti-TFEB antibody (Biolegend, United States, 1:100), anti-TFE3 antibody (Abnova, 1:50), anti-PKD1 antibody (Abnova, 1:80), anti-PKD3 antibody (Sigma-Aldrich, United States 1:100), anti-HDAC4 antibody (Cell Signaling United Kingdom, 1:100), anti-HDAC5 antibody (Cell Signaling United Kingdom, 1:100), and anti-HDAC7 antibody (Cell Signaling United Kingdom, 1:100). Alexa fluor 488 Goat Anti-Mouse IgG (H + L) (Life Technologies, United States, 1:1,000) and Alexa fluor 555 Goat Anti-Rabbit IgG (H + L) (Life Technologies, United States, 1:1,000) were used as secondary antibodies. 4′,6-diamidino-2-phenylindole, dihydrochloride (300 mM, Thermo Fisher Scientific, United States) was used to stain for nuclei. Immunostained cells were embedded

TABLE 1 | Primers used for generation of cDNA expression plasmids.

Primer	Sequence
TFE3 pcDNA3.1-N-FLAG forward (*ClaI*)	5′-CCA TCG ATT CTC ATG CAG CCG AGC-3′
TFE3 pcDNA3.1-N-FLAG reverse (*XbaI*)	5′-GCT CTA GAT CAG GAC TCC TCT TCC ATG CT-3′
MiTF pcDNA3.1-N-FLAG forward (*ClaI*)	5′-CCA TCG ATC AGT CCG AAT CGG GAA TC-3′
MiTF pcDNA3.1-N-FLAG reverse (*XbaI*)	5′-GCT CTA GAC TAA CAC GCA TGC TCC GTT TC-3′
PKD2 pcDNA3.1-CA-N-MYC forward (*ClaI*)	5′-CCA TCG ATG CCG CCG CCC CCT CCC ATC CCG-3′
PKD2 pcDNA3.1-CA-N-MYC reverse (*EcoRI*)	5′-GAA TTC TCA GAG GAT GCT GAT GCG CTC AGC-3′
PKD2 pcDNA3.1-CA-N-FLAG forward (*EcoRI*)	5′-CGG AAT TCG CCG CCG CCC CCT CCC ATC C-3′
PKD2 pcDNA3.1-CA-N-FLAG reverse (*NotI*)	5′-ATA GTT TAG CGG CCG CCA GAG GAT GCT GAT GCG CTC-3′
PKD3 pcDNA3.1-CA-N-MYC forward (*ClaI*)	5′-CCA TCG ATT CTG CAA ATA ATT CCC CTC CA-3′
PKD3 pcDNA3.1-CA-N-MYC reverse (*EcoRI*)	5′-GAA TTC CTA AGG ATC CTC CTC CAT GT-3′
PKD3 pcDNA3.1-CA-N-FLAG forward (*XhoI*)	5′-CCG CTC GAG CTA AGG ATC CTC CTC CAT GTC G-3′
PKD3 pcDNA3.1-CA-N-FLAG reverse (*EcoRI*)	5′-CGG AAT TCT CTG CAA ATA ATT CCC CTC C-3′

in ProLong™ Gold antifade mountant (Life Technologies, United States). Images were acquired with a Zeiss LSM 700 confocal microscope and processed with ZEN 2009 (Zeiss) and Fiji software.

COS-7 cells were plated in six-well plates on sterile coverslips. At the experimental endpoint, cells were PBS-washed and fixed in 3.7% formaldehyde for 10 min at room temperature. Permeabilization and blocking of cells were carried out in a single step with 0.3% Triton X-100, 0.5% goat serum (Abcam) in PBS for 1 h at room temperature. Coverslips were incubated with the specific primary antibody at 4°C overnight. Secondary antibodies conjugated with Alexa Fluor® 488 or Alexa Fluor® 555 were diluted in PBS and incubated for 2 h at room temperature. 4′,6-diamidino-2-phenylindole, dihydrochloride (300 mM, Thermo Fisher Scientific, United States) was used to stain for nuclei. Immunostained cells were embedded in ProLong™ Gold antifade mountant (Invitrogen, United States). Images were acquired with a Zeiss LSM 700 confocal microscope and processed with ZEN 2009 (Zeiss) and Fiji software.

Protein Extraction and Western Blot Analysis

Western blot analysis was performed as recently published (Schmidt et al., 2014; Lodka et al., 2016; Huang et al., 2017; Zhu et al., 2017). Shortly, cells were lysed in ice-cold extraction buffer [10-mM Tris-hydrochloride, pH 7.5, 140-mM sodium chloride, 1-mM ethylenediaminetetraacetic acid, 25% glycerol, 0.5% sodium dodecyl sulfate (SDS), 0.5% Non-idet P-40, 0.1-mM dithiothreitol, 0.5-mM phenylmethylsulfonyl fluoride, and 100 ng/ml complete ethylenediaminetetraacetic acid-free protease inhibitor cocktail, ROCHE] and then cleared by centrifugation (4°C, 15 min, 12,000 × *g*). The Bio-Rad Protein Assay was used to quantitate protein concentration in the supernatant. Isolated proteins were frozen and stored at −80°C until usage. For Western blot analysis, 20 μg protein was separated by 10% SDS polyacrylamide gel electrophoresis and blotted onto polyvinylidene fluoride or nitrocellulose membranes (GE Healthcare, United Kingdom). Membranes were incubated with specific primary antibodies: anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, clone 6C5, Millipore, United States, 1:10,000), anti-HDAC4 antibody (Cell Signaling, United Kingdom, 1:1,000), anti-HDAC5 antibody (Cell Signaling, United Kingdom, 1:1,000), anti-HDAC7 antibody (Cell Signaling, United Kingdom, 1:1,000), anti-phospho-HDAC4 (Ser246)/HDAC5 (Ser259)/HDAC7 (Ser155) (Cell Signaling United Kingdom, 1:1,000), anti-phospho-HDAC4 (Ser632)/HDAC5 (Ser661)/HDAC7 (Ser486) (Cell Signaling United Kingdom, 1:1,000), anti-DYKDDDDK (FLAG-tag, Cell Signaling United Kingdom, 1:1,000), and anti-MYC (Millipore, 1:500) and secondary horseradish peroxidase-conjugated antibodies: anti-mouse IgG (Cell Signaling, United Kingdom, 1:3,000), anti-rabbit IgG (Cell Signaling, United Kingdom, 1:20,000), and anti-goat IgG (Abcam, United Kingdom, 1:3,000). The signal™ was visualized with Super Signal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, United States).

Co-immunoprecipitation

Transfected cells were washed with ice-cold PBS and resuspended in lysis buffer (50 mM potassium phosphate, 150 mM sodium chloride, 0.5% Triton X-100, pH 7.4). Lysates were cleared by centrifugation at 10,000 × *g* for 10 min at 4°C. For co-immunoprecipitation, supernatants were incubated with 30 μl of prewashed anti-FLAG M2 agarose (Sigma, A2220) for 2 h at 4°C. Immunoprecipitated proteins were eluted from agarose by 5 min boiling at 95°C in Laemmli sample buffer (125-mM Tris-hydrochloride, 10% glycerol, 10% SDS, 130-mM dithiothreitol) and analyzed by Western blot.

Statistics

All experiments were performed independently and at least three times using biological triplicates each and technical duplicates for each biological replicate. Data are shown as mean ± SEM. For all comparisons, one-way analyses of variance (ANOVAs) followed by a pairwise Student's *t*-test for independent groups were performed using GraphPad Prism 8.3.0 (GraphPad Software, Inc.) and Excel 2016 (Microsoft) software. *P*-values of less than 0.05 were considered statistically significant ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). Adobe Illustrator, version 16.0.0, was used for illustrations.

RESULTS

Class IIa Histone Deacetylases 4, 5, and 7 Inhibit Transcription Factor EB-Mediated *Tripartite Motif-Containing 63* Expression

Previously, we showed that TFEB increases the expression of human *TRIM63* by binding to specific E-box elements close to the transcription start site in its promoter (Du Bois et al., 2015). We showed that TFEB transcriptional activity was attenuated by HDAC5 that physically interacted and colocalized with TFEB in myocytes *in vitro* (Du Bois et al., 2015). Because HDAC4 and HDAC5 collectively inhibit the activity of the bHLH TF myogenin toward *TRIM63* expression (Moresi et al., 2010), we hypothesized that other class IIa HDACs reduce the activity of TFEB on the *TRIM63* promoter as well. Using luciferase assays, we found that HDAC4 (**Figure 1A**), HDAC5 (**Figure 1B**), and HDAC7 (**Figure 1C**) inhibit TFEB-induced activity of the human *TRIM63* luciferase reporter in a dose-dependent manner. These data indicate that TFEB-induced *TRIM63* expression is inhibited by not only HDAC5 but also HDAC4 and HDAC7.

Protein Kinase D Family Members Attenuate Histone Deacetylase-Mediated Inhibition of Transcription Factor EB-Induced *Tripartite Motif-Containing 63* Expression

Earlier, we reported that PKD1 associates with phosphorylates and facilitates 14-3-3-mediated nuclear export of HDAC5, which relives inhibition of TFEB and thereby increases *TRIM63*

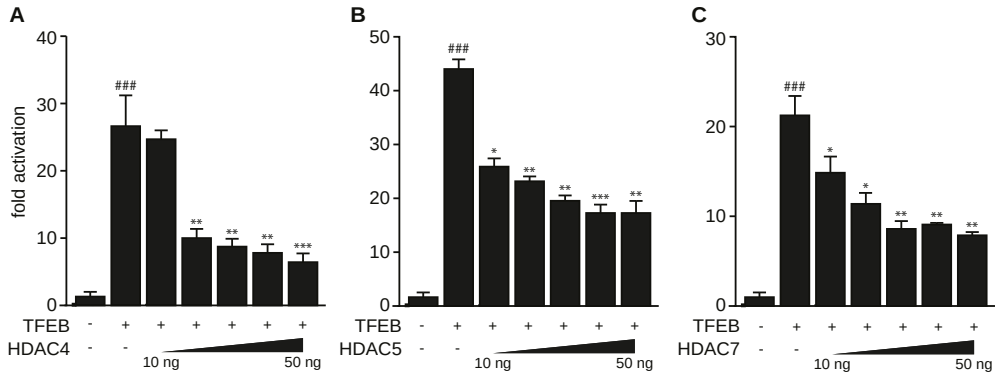

FIGURE 1 | Class IIa histone deacetylases 4, 5, and 7 inhibit TFEB-induced TRIM63 expression. **(A–C)** Luciferase assays performed with cell extracts of COS-7 cells transfected with Hs_*TRIM63*-Luc (-543 bp), FLAG-TFEB (TFEB) and increasing amounts of **(A)** MYC-HDAC4 (HDAC4), **(B)** MYC-HDAC5 (HDAC5), or **(C)** MYC-HDAC7 (HDAC7) as indicated or control (-) plasmid. Luciferase activity was normalized to expression of CMV-LacZ and expressed as fold increase. Data are represented as mean ± SEM. One-way ANOVA $P < 0.0001$ for **(A–C)**; $^{###}P < 0.005$ vs. control transfection with pcDNA3.1; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ vs. transfection with TFEB only.

expression (Du Bois et al., 2015). Because the three PKD-family members PKD1, PKD2, and PKD3 share a high degree of similarity in their functional domains (Avkiran et al., 2008) and were shown to phosphorylate HDAC5 (Huynh and McKinsey, 2006), we hypothesized that the PKD-family regulates TFEB activity by inactivating HDAC4, HDAC5, and HDAC7 as well. To investigate the effect on HDAC-mediated inhibition of TFEB, we performed luciferase assays. As already shown, TFEB-induced *TRIM63* activity was inhibited by HDAC4 (**Figure 2A**), HDAC5 (**Figure 2B**), and HDAC7 (**Figure 2C**). In contrast, the class I HDACs 1 and 3 did not decrease TFEB-induced *TRIM63* activity (**Supplementary Figure 1A**). When we cotransfected PKD1 (**Figures 2A–C**, left panels), PKD2 (**Figures 2A–C**, middle panels), or PKD3 (**Figures 2A–C**, right panels), the repressive effects of all three HDACs were relieved. CamK IV, which was used as a positive control, also relieved the repressive effects of HDAC4, HDAC5, and HDAC7 in the same assay (**Supplementary Figure 2A**). Our data indicate that the PKD family converges on HDAC4, HDAC5, and HDAC7 to control *TRIM63* expression. To investigate if the observed effects of the PKD family onto HDAC-mediated inhibition of TFEB are due to nuclear export of HDAC4 and HDAC7, we performed immunocytochemistry experiments. As expected, we observed colocalization of HDAC5 (**Figure 2D**, left panel) and also HDAC4 (**Figure 2D**, middle panel) and HDAC7 (**Figure 2D**, right panel) with TFEB in the nucleus. This colocalization is a possible molecular basis for class IIa HDAC-mediated TFEB inhibition. When we cotransfected with PKD1, HDAC4, HDAC5, and HDAC7 translocated to the cytoplasm, whereas TFEB remained in the nucleus (**Figure 2D**).

Phosphoserines 246, 467, and 632 in HDAC4 (Backs et al., 2006), 259 and 498 in HDAC5 (Zhang et al., 2002), and 155, 181, 321, and 449 in HDAC7 (Dequiedt et al., 2005) serve as binding sites for the chaperone protein 14-3-3 and are known PKD1 phosphorylation sites. Previously, we confirmed that PKD1 binds to HDAC5 and phosphorylates its 14-3-3 consensus sites. To investigate if PKD1 has a similar effect on HDAC4 and HDAC7, we performed a mammalian-two-hybrid assay using GAL4 upstream activator sequence (UAS)-luciferase, as published recently (Chang et al., 2005; Du Bois et al., 2015). This assay was also used to investigate if PKD2 phosphorylates HDAC4, HDAC5, and HDAC7 as well. In this assay, the N-terminus of HDAC4, HDAC5, or HDAC7 is fused to the GAL4 DNA-binding domain, and 14-3-3 is fused to the VP16 transactivation domain. Under normal growth conditions, these HDACs are not phosphorylated in COS-7 cells. Thus, GAL4-HDAC4, GAL4-HDAC5, or GAL4-HDAC7 cannot interact with 14-3-3-VP16, and the GAL4-dependent UAS-luciferase cannot be activated (**Figure 3A**). Expression plasmids encoding these fusion proteins, together with UAS-luciferase, were transfected into COS-7 cells together with increasing amounts of PKD1 or PKD2 expression plasmids. PKD1 and PKD2 increased UAS-luciferase activity in a dose-dependent manner. These data indicate that PKD1 and PKD2 create phospho-14-3-3 recognition motifs in HDAC4, HDAC5, and HDAC7, which recruit 14-3-3 proteins that mediate their nuclear export.

We had identified amino acids 360-601 as PKD1 binding region in HDAC5 (Du Bois et al., 2015). To test if PKD2 and HDAC5 physically interact as well, we performed co-immunoprecipitation experiments with PKD2 and wild-type and deletion mutants of HDAC5. We found that PKD2 interacted avidly with HDAC5 and that amino acids 360–601 in HDAC5 were responsible for this interaction (**Figures 3B,C**).

To investigate if PKD1, PKD2, and PKD3 phosphorylate endogenous HDAC4, HDAC5, and HDAC7 in myocytes, we transfected the respective PKD expression plasmids into C2C12 cells and performed Western blot analyses on proteins isolated from these cells using anti-phospho-HDAC4 (Ser246)/HDAC5 (Ser259)/HDAC7 (Ser155), anti-phospho-HDAC4 (Ser632)/HDAC5 (Ser661)/HDAC7 (Ser486), anti-HDAC4, anti-HDAC5, anti-HDAC7, and anti-glyceraldehyde-3-phosphate dehydrogenase antibodies. PKD1, PKD2, and PKD3 phosphorylated all three endogenous class IIa HDACs in C2C12 cells (**Supplementary Figure 3A**).

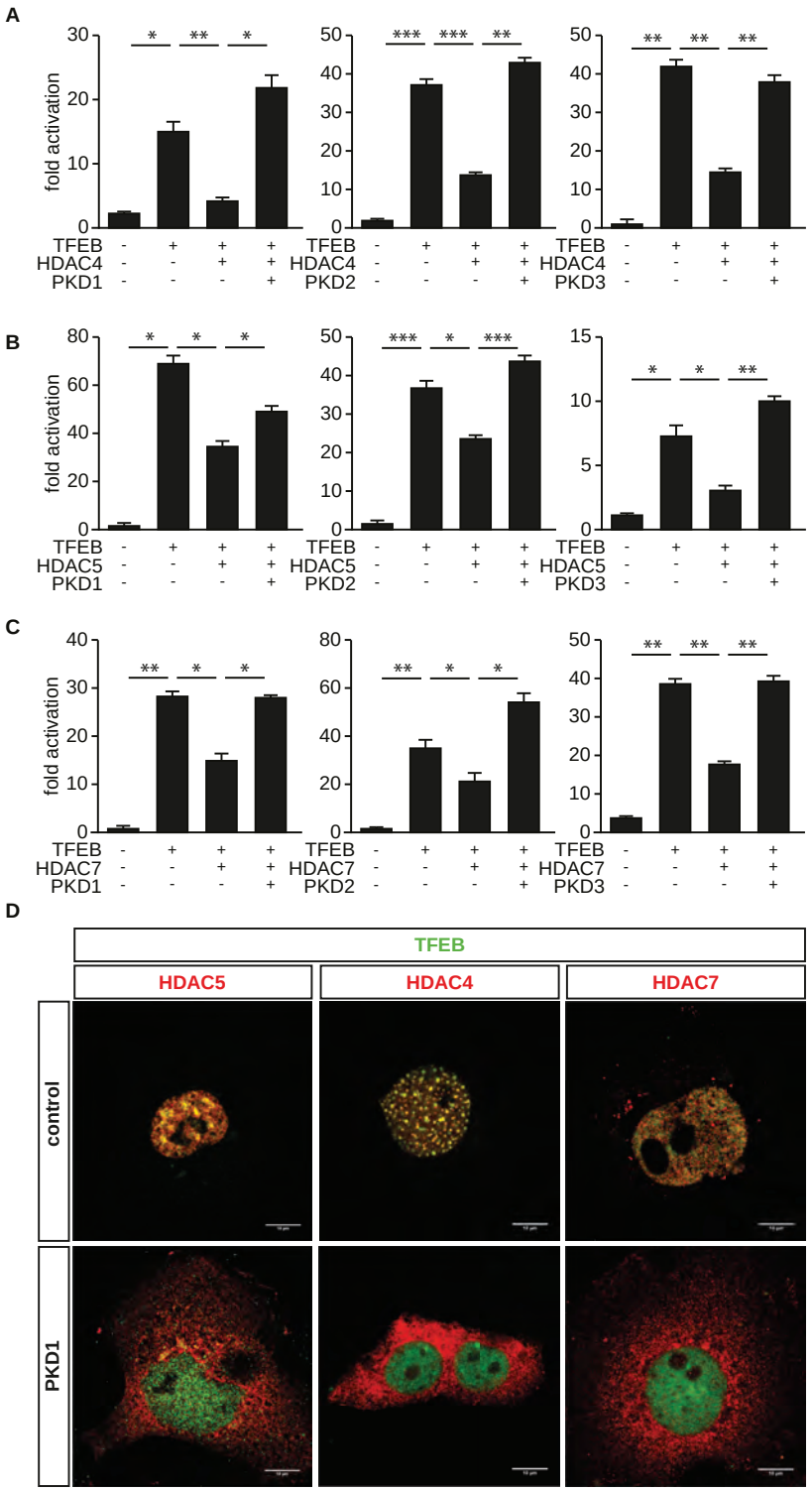

FIGURE 2 | Protein kinase D family members attenuate HDAC-mediated inhibition of TFEB-induced *TRIM63* expression. **(A–C)**, COS-7 cells were transfected with expression plasmids encoding FLAG-TFEB, **(A)** HDAC4-MYC, **(B)** HDAC5-MYC, or **(C)** HDAC7-MYC, or constitutively active (ca) PKD1 (left panel), caPKD2 (middle panel), and caPKD3 (right panel) proteins, as indicated, together with the Hs_*TRIM63*_Luc reporter construct (-543 bp). Values were normalized to expression of CMV-LacZ and calculated as the fold increase in luciferase/CMV-LacZ ratio compared with the reporter alone. Data are represented as mean ± SEM. One-way ANOVA $P < 0.0001$ for **(A–C)**; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$. $n = 5$. **(D)** COS-7 cells were transfected with FLAG-TFEB and HDAC4-MYC, HDAC5-MYC, and HDAC7-MYC together with a pcDNA3.1 control vector or caPKD1, as indicated. Immunostaining was performed with anti-FLAG (green) and anti-MYC (red) antibodies. Scale bars, 10 μm.

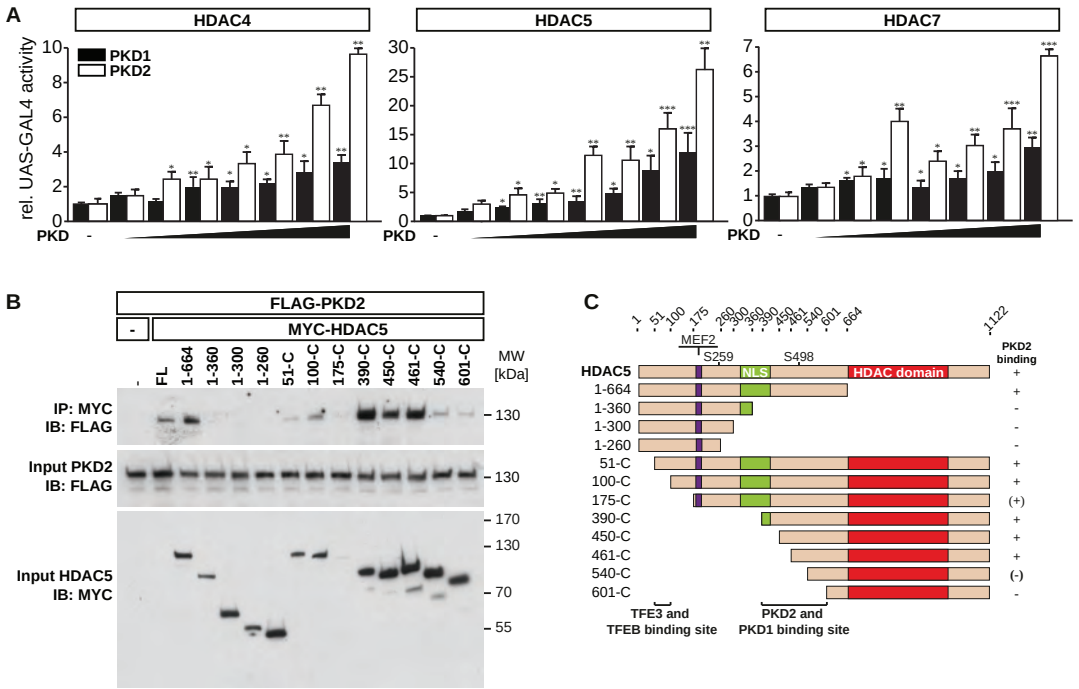

FIGURE 3 | PKD1 and PKD2 mediate phosphorylation and 14-3-3 binding of class IIa HDACs. **(A)** COS-7 cells were transfected with upstream activator sequence (UAS)-luciferase and expression plasmids encoding GAL4 fused with the wild-type (WT) HDAC4 (left panel), HDAC5 (middle panel), or HDAC7 (right panel) N-terminal extension together with 14-3-3-VP16 and increasing amounts of expression plasmids of constitutively active (ca) PKD1 or caPKD2 (from 6.25 to 400 ng), as indicated. Values were normalized to expression of CMV-LacZ and calculated as fold increase. Data are represented as mean ± SEM. One-way ANOVA $P < 0.0001$ for all; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$. $n = 3$. **(B)**, Co-immunoprecipitation (Co-IP) assay with lysates from COS-7 cells expressing FLAG-PKD2 and deletion mutants of MYC-HDAC5, as indicated. HDAC5 fusion proteins were immunoprecipitated (IP) with anti-MYC antibodies, and PKD2 fusion proteins were detected with an antibody directed against FLAG. Input proteins were detected by Western blot (immunoblot, IB) with antibodies directed against the FLAG- or MYC-tag. $n = 3$. FL indicates full length. **(C)** Scheme of PKD2 binding site in HDAC5.

We used the pan-PKD inhibitor CID 2011756 to investigate the specificity of PKD1-mediated HDAC4-, HDAC5-, and HDAC7-phosphorylation in myocytes. C2C12 myocytes were transfected with PKD1 or pcDNA and treated with CID 2011756 (50 μM) or vehicle (dimethyl sulfoxide) for 6 h. Western blot analyses of protein lysates showed that CID 2011756 attenuated PKD1-mediated phosphorylation of all three endogenous class IIa HDACs in myocytes (**Supplementary Figure 3B**). In summary, these data indicate that PKD1, PKD2, and PKD3 attenuate the inhibitory effect of HDAC4, HDAC5, and HDAC7 onto TFEB-induced *TRIM63* expression by phosphorylation and 14-3-3 mediated nuclear export of these HDACs.

Transcription Factor Binding to Immunoglobulin Heavy-Chain Enhancer 3 but Not Microphthalmia-Associated Transcription Factor Increase *Tripartite Motif-Containing 63* Expression

Because TFEB and the closely related MiTF-family members TFE3 and MiTF elicit some degree of functional redundancy and bind to the same E-box elements (Steingrimsson et al., 2004; Martina et al., 2014; Pastore et al., 2016), we hypothesized that TFE3 and MiTF could regulate *TRIM63* expression as well. Indeed, TFEB (**Figure 4A**) and TFE3 (**Figure 4B**) but not MiTF (**Figure 4C**) increased *TRIM63* activity in a dose-dependent manner. Thus, for further experiments, we focused on TFE3 and excluded MiTF. Using the wild-type and E-box mutated *TRIM63* reporter constructs (**Figure 4D**; Du Bois et al., 2015), we found that the TFE3-induced *TRIM63* expression was strongly reduced in E-box 1 and E-box 3 mutants, whereas mutation of E-box 2 had a weaker effect (**Figure 4E**). These data indicate that E-box 1 and E-box 3 mediate the TFEB- and TFE3-induced *TRIM63* activity.

Transcription Factor EB and Transcription Factor Binding to Immunoglobulin Heavy-Chain Enhancer 3 Strongly Interact With Each Other

Because TFEB and TFE3 used the same E-box motives, we investigated if TFEB and TFE3 have additive functions on the *TRIM63* promoter. We found that equimolar concentrations of TFEB and TFE3 activated the *TRIM63* promoter to comparable degrees and that their combination did not result in a

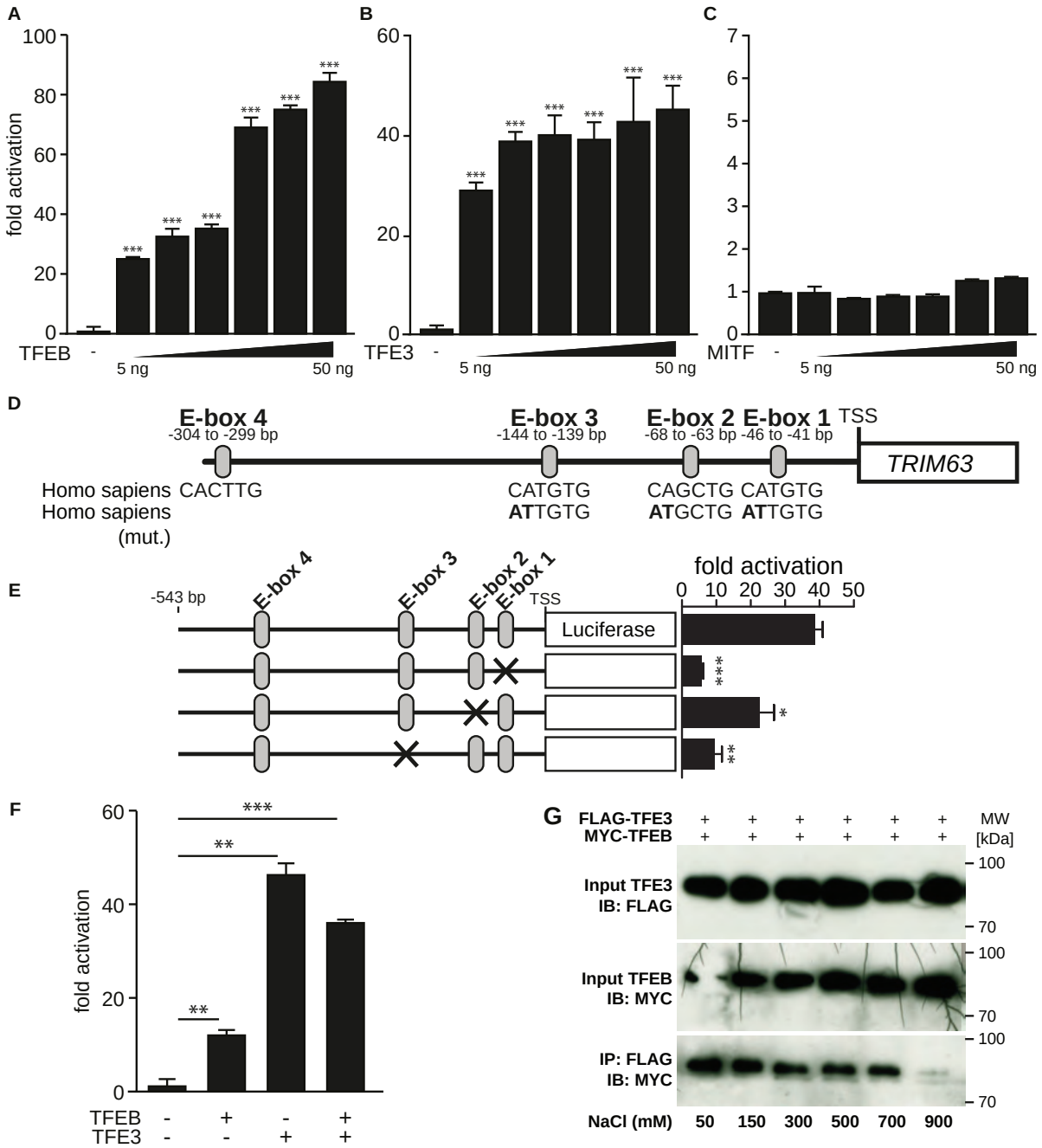

FIGURE 4 | MiTF/TFE family member TFE3 but not MiTF increase *TRIM63* expression. **(A–C)** Luciferase assays performed on cell extracts of COS-7 cells transfected with Hs_*TRIM63*-Luc (-543 bp) with increasing amounts of **(A)** FLAG-TFEB (TFEB), **(B)** FLAG-TFE3, or **(C)** FLAG-MiTF as indicated or control (-) plasmid. Luciferase activity was normalized to expression of CMV-LacZ and expressed as fold increase. Data are represented as mean ± SEM. ***$P < 0.005$ vs. control transfection with pcDNA3.1. **(D)** Schematic diagram of the human *TRIM63*-promoter. Positions of conserved E-box motifs (CANNTG) relative to the transcription start site (TSS) are indicated. *Homo sapiens* (mut.) indicates mutated nucleotides to inactivate individual E-boxes (mutated nucleotides are shown in bold). One-way ANOVA $P < 0.0001$ **(A,B)** and n.s. **(C)**, respectively; *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$. **(E)** COS-7 cells were transfected with a TFE3 expression plasmid and the indicated *TRIM63*-promoter constructs (–543 bp) harboring E-box mutations, as shown in **(D)**. Data are represented as mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$. **(F)** Luciferase assays (Hs_*TRIM63*-Luc; -543 bp) performed on cell extracts of COS-7 cells after a single transfection with either MYC-TFEB (TFEB) or FLAG-TFE3 (TFE3) or cotransfection with MYC-TFEB (TFEB) and FLAG-TFE3 (TFE3). Luciferase activity was normalized to expression of CMV-LacZ and expressed as fold increase. Data are represented as mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$. **(G)**, Co-immunoprecipitation (Co-IP) assay with lysates from COS-7 cells expressing FLAG-TFE3 and MYC-TFEB. TFE3 fusion proteins were immunoprecipitated (IP) with anti-FLAG M2 agarose, and TFEB fusion proteins were detected with an antibody directed against MYC. Input proteins were detected by Western blot (immunoblot, IB) with antibodies directed against the FLAG- or MYC-tag. $n = 3$. During cell lysis and IP increasing concentrations of sodium chloride (50, 100, 300, 500, 700, and 900 mM) were used as indicated.

further increase in *TRIM63* activity (**Figure 4F**). These data indicate that TFEB and TFE3 are possibly competing for the same E-box elements. As TFEB and TFE3 are known to heterodimerize, we tested the strength of this interaction. We performed cell lysis and co-immunoprecipitation with increasing sodium chloride buffer concentrations ranging from 50- to 900-mM sodium chloride. Only the highest sodium chloride concentration used attenuated the interaction between TFEB and TFE3, indicative of a strong interaction of both proteins (**Figure 4G**).

Class IIa Histone Deacetylases 4, 5, and 7 Inhibit Transcription Factor Binding to Immunoglobulin Heavy-Chain Enhancer 3-Mediated *Tripartite Motif-Containing 63* Expression

We next hypothesized that the activity of TFE3 on the *TRIM63* promoter is also inhibited by class IIa HDACs. Using luciferase assays, we found that HDAC4 (**Figure 5A**), HDAC5 (**Figure 5B**), and HDAC7 (**Figure 5C**) inhibited TFE3-induced *TRIM63*

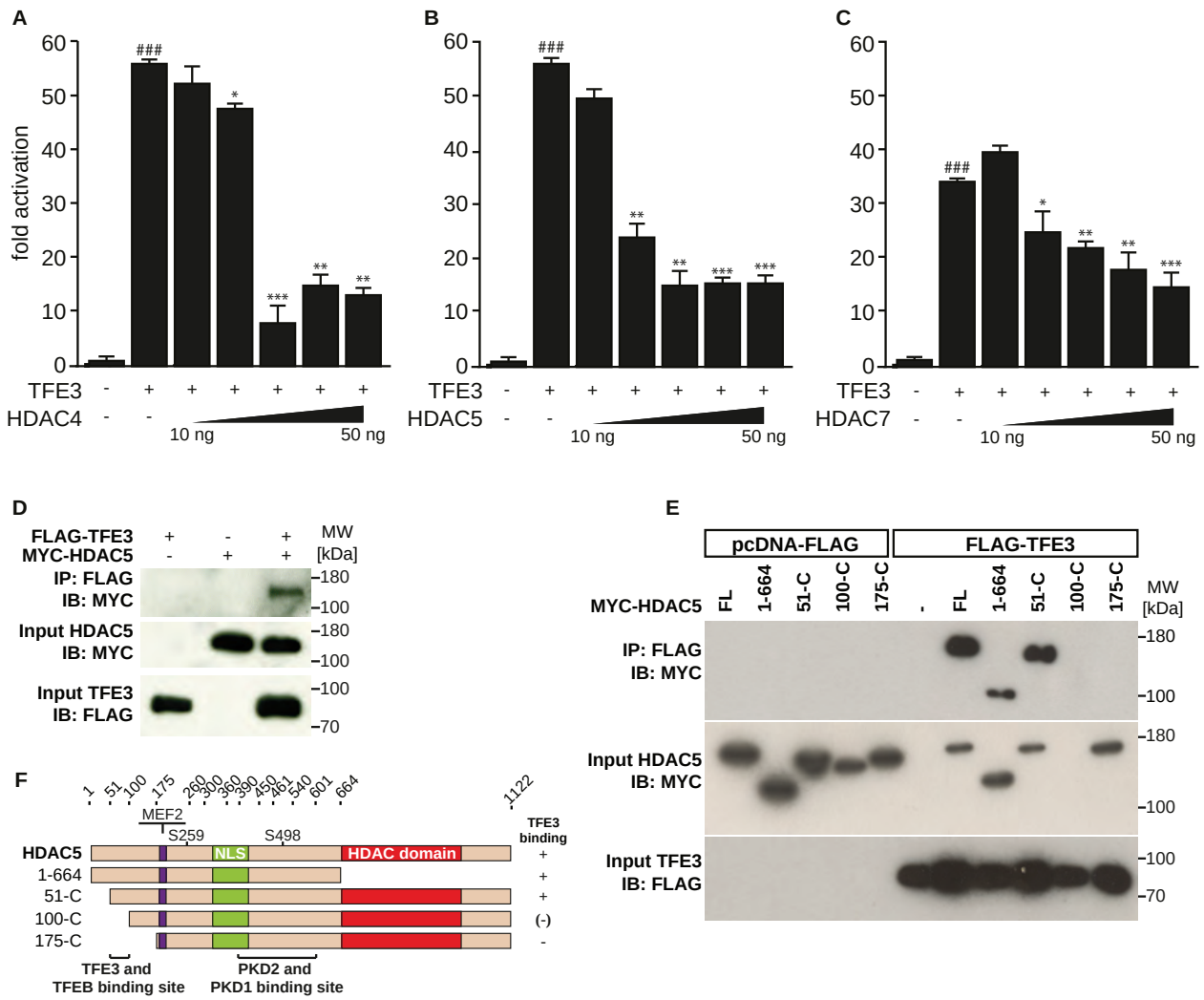

FIGURE 5 | Class IIa histone deacetylases 4, 5, and 7 inhibit TFE3-mediated *TRIM63* expression. **(A–C)**, Luciferase assays performed on cell extracts of COS-7 cells transfected with Hs_*TRIM63*-Luc (-543 bp), FLAG-TFE3 (TFE3), and increasing amounts of **(A)** MYC-HDAC4 (HDAC4), **(B)** MYC-HDAC5 (HDAC5), or **(C)** MYC-HDAC7 (HDAC7) as indicated or control (-) plasmid. Luciferase activity was normalized to expression of CMV-LacZ and expressed as fold increase. Data are represented as mean ± SEM. One-way ANOVA $P < 0.0001$ for **(A–C)**; $^{###}P < 0.005$ vs. control transfection with pcDNA3.1; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ vs. transfection with TFE3 only. **(D)** Co-IP assay with lysates from COS-7 cells expressing FLAG-TFE3 and MYC-HDAC5. TFE3 fusion proteins were immunoprecipitated (IP) with anti-FLAG M2 agarose, and HDAC5 fusion proteins were detected with an antibody directed against MYC. Input proteins were detected by Western blot (immunoblot, IB) with antibodies directed against the FLAG- or MYC-tag. $n = 3$. **(E)** Co-IP assay with lysates from COS-7 cells expressing FLAG-TFE3 and deletion mutants of MYC-HDAC5, as indicated. TFE3 fusion proteins were immunoprecipitated (IP) with anti-FLAG M2 agarose, and HDAC5 fusions proteins were detected with an antibody directed against MYC. Input proteins were detected by Western blot (immunoblot, IB) with antibodies directed against the FLAG- or MYC-tag. $n = 3$. **(F)**, Scheme of TFE3 binding site in HDAC5.

activity in a dose-dependent manner, indicating that not only TFEB but also TFE3 is collectively controlled by class IIa HDACs. In contrast, TFE3-induced *TRIM63* activity was not reduced by HDAC1 or HDAC3 (**Supplementary Figure 1B**). We performed co-immunoprecipitation experiments and found that TFE3 and HDAC5 interacted with each other (**Figure 5D**). We next performed co-immunoprecipitation experiments with TFE3 and wild-type and deletion mutants of HDAC5 to determine which region within HDAC5 was required for its interaction with TFE3. We found that amino acids 51–175 of HDAC5 are required for physical interaction with TFE3 and, therefore, define a TFE3 binding site (**Figures 5E,F**). We performed immunocytochemistry using antibodies directed against endogenous TFE3 and endogenous HDAC4, HDAC5, and HDAC7 and found that these proteins colocalized in C2C12 myocytes indicating that TFE3 interacts with class IIa HDACs in myocytes (**Supplementary Figure 4**).

Protein Kinase D Family Members Attenuate Histone Deacetylase-Mediated Inhibition of Transcription Factor Binding to Immunoglobulin Heavy-Chain Enhancer 3-Induced *Tripartite Motif-Containing 63* Activity

Based on our findings, we hypothesized that the PKD-family members could abolish the inhibitory effects of HDAC4, HDAC5, and HDAC7 toward TFE3. To test this hypothesis, we performed luciferase assays and found that the TFE3-induced *TRIM63* activity was inhibited by HDAC4 (**Figure 6A**), HDAC5 (**Figure 6B**), and HDAC7 (**Figure 6C**). Importantly, cotransfection of PKD1 (**Figure 6**, left panel), PKD2 (**Figure 6**, middle panel), or PKD3 (**Figure 6**, right panel) relieved the repressive effects of all three HDACs. CamK IV also relieved the repressive effects of HDAC4, HDAC5, and HDAC7 in the same assay (**Supplementary Figure 2B**). To investigate if the observed effects of the PKD family onto HDAC-mediated inhibition of TFE3 are due to nuclear export of HDAC4, HDAC5, or HDAC7, we performed immunocytochemistry experiments. We observed colocalization of all class IIa HDACs with TFE3 in the nucleus (**Figure 6D**). When we cotransfected with PKD1, HDAC5 (**Figure 6D**, left panel), HDAC4 (**Figure 6D**, middle panel), and HDAC7 (**Figure 6D**, right panel) translocated to the cytoplasm, whereas TFE3 remained in the nucleus. These data show that PKD1, PKD2, and PKD3 attenuated the inhibitory effect of HDAC4, HDAC5, and HDAC7 onto TFE3-induced *TRIM63* expression by nuclear export of these HDACs.

DISCUSSION

We report that *TRIM63* expression is tightly regulated by a transcriptional network comprised of the PKD-family members PKD1, PKD2, and PKD3, the class IIa HDACs HDAC4, HDAC5, and HDAC7, as well as the MiT/TFE family members TFEB and TFE3. We show that HDAC4, HDAC5, and HDAC7 colocalize with TFEB and TFE3 in the nucleus of non-muscle cells and inhibit TFEB- and TFE3-induced *TRIM63* expression. We demonstrate that PKD1, PKD2, and PKD3 phosphorylate HDAC4, HDAC5, and HDAC7. This state-of-affairs facilitates their binding to the 14-3-3 chaperon protein and their consecutive nuclear-export relieving repression of TFEB- and TFE3-induced *TRIM63* expression. All PKD family members ameliorated HDAC-mediated inhibition of TFEB- and TFE3-mediated *TRIM63* promoter activity. We conclude that *TRIM63* expression, which is a key factor in UPS-dependent protein degradation in skeletal muscle during atrophy, is regulated at multiple levels.

Skeletal muscle atrophy is characterized by a reduction in myofiber size, mainly due to a net loss of structural proteins leading to a reduced muscle mass and function. As it occurs in many pathological conditions, such as critical illness and inflammation, this results from increased UPS-dependent protein degradation (Hershko and Ciechanover, 1998; Ciechanover, 2006). However, muscle atrophy is a complex process that is mediated by multiple factors. Indeed, the molecules, mediators, and cellular pathways contributing to muscle atrophy are still being discovered (Bodine and Baehr, 2014). In 2001, Bodine et al. (2001a) identified *TRIM63*/MuRF1 as an atrogene that was highly upregulated in atrophying muscle after denervation, immobilization, and unweighting-induced atrophy in rats. MuRF1/*Trim63* knockout mice showed less denervation-induced muscle atrophy compared with wild-type mice (Bodine et al., 2001a). Thereafter, we and others confirmed the involvement of MuRF1 in muscle atrophy (Du Bois et al., 2015), described its increased expression in physiological and pathological muscle atrophy (Schmidt et al., 2014; Wollersheim et al., 2014; Huang et al., 2017), and discovered MuRF1 targets (Fielitz et al., 2007; Witt et al., 2008; Labeit et al., 2010; Nowak et al., 2019).

Increased *TRIM63* expression is a well-established and accepted read-out for skeletal muscle atrophy in general. The expression of MuRF1/*TRIM63* is regulated by several signaling pathways converging onto multiple TFs, such as the forkhead box protein family (Stitt et al., 2004; Raffaello et al., 2010) and myogenin (Moresi et al., 2010). Glass and co-workers demonstrated that the insulin-like growth factor/phosphoinositide 3-kinase/protein kinase B/Akt pathway, which had previously been shown to induce hypertrophy (Bodine et al., 2001b), suppresses atrophy by downregulating MuRF1. Myogenin was also shown to regulate *TRIM63*, and mice that lack myogenin failed to upregulate *TRIM63* expression. These mice were resistant to denervation-induced muscle atrophy (Moresi et al., 2010). Given the importance of transcriptional regulation of *TRIM63,* we previously performed a cDNA expression screen to identify regulators of *TRIM63* expression. We identified TFEB as an important TF and described its regulation and importance in muscle atrophy in myocytes *in vitro* and in muscle *in vivo* (Du Bois et al., 2015).

TFEB belongs to the MiT/TFE family of bHLH-LZ TFs, including TFE3, MiTF, and TFEC (Steingrimsson et al., 2004). All MiT/TFE TFs recognize a unique E-box motif within the

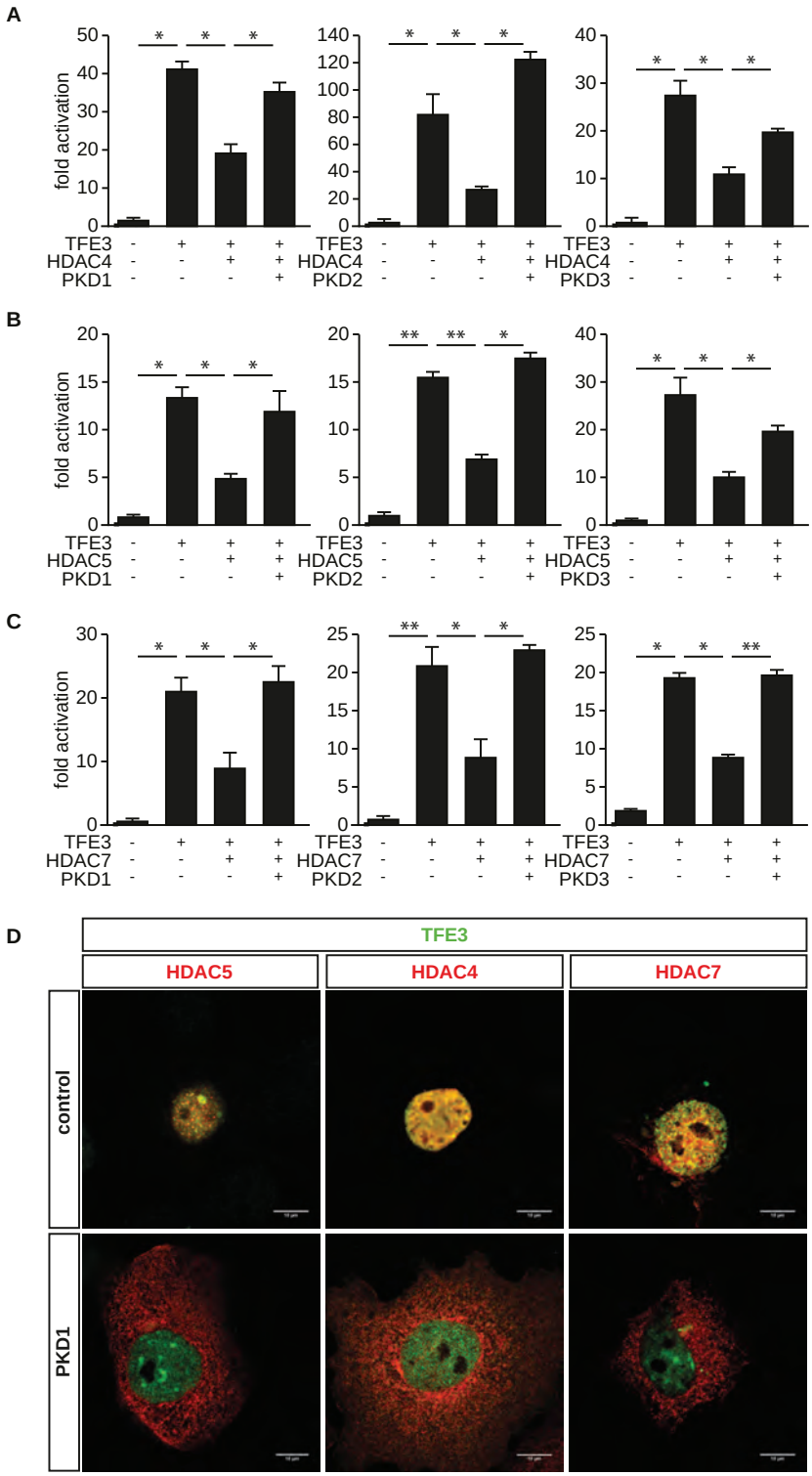

FIGURE 6 | Protein kinase D family members attenuate HDAC-mediated inhibition of TFE3-induced *TRIM63* expression. **(A–C)**, COS-7 cells were transfected with expression plasmids encoding FLAG-TFE3, **(A)** HDAC4-MYC, **(B)** HDAC5-MYC, or **(C)** HDAC7-MYC, or constitutively active (ca) PKD1 (left panel), caPKD2 (middle panel), and caPKD3 (right panel) proteins, as indicated, together with the Hs_*TRIM63*_Luc reporter construct (-543 bp). Values were normalized to expression of CMV-LacZ and calculated as the fold increase in luciferase/CMV-LacZ ratio compared with the reporter alone. Data are represented as mean ± SEM. One-way ANOVA $P < 0.0001$ for **(A–C)**; $^{*}P < 0.05$; $^{**}P < 0.005$. $n = 5$. **(D)** COS-7 cells were transfected with FLAG-TFE3 and HDAC4-MYC, HDAC5-MYC, and HDAC7-MYC together with an empty vector (pcDNA_3.1) or caPKD1, as indicated. Immunostaining was performed with anti-FLAG (green) and anti-MYC (red) antibodies. Scale bars, 10 μm.

proximal promoters of lysosomal and autophagy genes (Sardiello et al., 2009; Palmieri et al., 2011; Martina et al., 2014) and regulate cellular catabolism and nutrient-dependent lysosomal response (Settembre et al., 2011; Slade and Pulinilkunnil, 2017). Within the MiT/TFE family, a close relationship exists between TFEB and TFE3. TFEB and TFE3 regulate cellular adaptation to stress by induction of lysosomal biogenesis and autophagy and immune responses, metabolism, mitochondrial homeostasis, and unfolded-protein responses. TFEB and TFE3 were shown to regulate the same genes (Pastore et al., 2016), such as *CDH1*, encoding E-cadherin (Huan et al., 2005), *CD40L*, encoding CD40 ligand (Huan et al., 2006), and *PPARGC1a*, encoding PGC1α (Settembre et al., 2013). However, whether or not TFE3 activates *TRIM63* expression was not known. We report that not only TFEB but also TFE3 regulates *TRIM63* expression and that both TFs are controlled by the PKD and HDAC enzyme families. To the well-described TFEB and TFE3 functions, we add that both TFs also regulate *TRIM63* expression. We propose that they activate UPS- and autophagy-mediated protein degradation. We showed earlier that TFEB regulates *TRIM63* expression via binding to E-box elements in the *TRIM63* promoter, and mutation of these binding motifs abrogated TFEB-dependent *TRIM63* expression. However, although MiT/TFE family members bind specific E-box elements and use the same E-box motifs as TFEB to induce *TRIM63* expression, we have not yet demonstrated direct binding of TFE3 to the native *TRIM63* promoter. Such analyses need to be performed.

MuRF1 and the regulation of its expression are important for metabolic adaptations of striated muscles (Hirner et al., 2008; Witt et al., 2008; Rudolf et al., 2013). MuRF1 mediates the degradation of numerous metabolic enzymes, such as pyruvate dehydrogenase and its regulator pyruvate dehydrogenase kinase that are key mitochondrial enzymes regulating glycolysis. Interaction studies also demonstrated that MuRF1 interacts with pyruvate kinase 2, phosphorylase beta, and glycogenin, which are involved in glycolysis and glycogen metabolism, respectively (Hirner et al., 2008). Because TFEB (Mansueto et al., 2017), TFE3 (Iwasaki et al., 2012), and forkhead box (Schiaffino et al., 2013) are also involved in muscle metabolism and mitochondrial homeostasis, these effects are possibly mediated by their ability to regulate *TRIM63* expression. As our conclusions are mainly based on data obtained from non-muscle cells, this hypothesis warrants further investigation to show its relevance in muscle metabolism.

Distinguished by their structures and expression patterns, HDACs can be divided into different classes. We focused on the heart and skeletal muscle enriched class IIa HDACs 4, 5, and 7. All class IIa HDACs repress the activity of myocyte enhancer factor 2 (MEF2), which reduces the expression of MEF2 target genes and suppresses the formation of slow-twitch, oxidative fibers in the muscle (Lu et al., 2000a,b; McKinsey et al., 2000; Zhang et al., 2002; Potthoff et al., 2007). HDAC4 and HDAC5 coordinately inhibited the activity of myogenin that is important for myogenesis (Moresi et al., 2010). Latter data implicated that HDAC4, HDAC5, and HDAC7 predominantly inhibit bHLH TFs contained in muscle to regulate muscular stress response. However, if other class IIa HDACs inhibit TFEB and if they can also reduce TFE3-mediated *TRIM63* expression was unknown. We describe that HDAC4, HDAC5, and HDAC7 inhibit TFEB-mediated *TRIM63* activity and show that all three HDACs inhibited TFE3. We propose that class IIa HDACs collectively control *TRIM63* expression. However, as our experiments were mainly performed in non-myocytes, further studies are needed to investigate if this mode of action also occurs in myocytes.

Protein function is often regulated by reversible protein phosphorylation by protein kinases, such as the PKD family (Rykx et al., 2003; Rozengurt, 2011). This family of stress-responsive serine-threonine kinases of the calmodulin-calcium-dependent-kinase family (termed PKD1, PKD2, and PKD3) play important roles in cell growth, differentiation, migration, and apoptosis (Rozengurt et al., 2005). Importantly, we and others showed that PKD1 mediates muscle atrophy (Du Bois et al., 2015), facilitates slow-twitch-fiber transformation in the muscle (Kim et al., 2008), and mediates cardiac stress response (Fielitz et al., 2008). However, the close structural relationship of the PKD family members especially in their functional domains (Avkiran et al., 2008) suggested that they might phosphorylate overlapping targets and might be involved in the observed phenotypes as well. Indeed, PKD1, PKD2, and PKD3 were all shown to phosphorylate HDAC5 (Huynh and McKinsey, 2006). This situation, in turn, relieves the repression of TFs such as MEF2 and TFEB (Fielitz et al., 2008; Du Bois et al., 2015). The mechanism is involved in the pathophysiology of cardiomyocyte hypertrophy *in vitro* and cardiac hypertrophy and remodeling *in vivo* (Vega et al., 2004; Fielitz et al., 2008). These observations suggested that PKD family members redundantly control the activity of class IIa HDACs (Huynh and McKinsey, 2006). However, it was uncertain if this interaction affects TFEB- or TFE3-mediated *TRIM63* expression. We confirmed this hypothesis for all three PKD family members and show that they phosphorylate HDAC4, HDAC5, and HDAC7 and promote their nuclear export. Because a high degree of functional redundancy was reported for PKD1 and PKD2, we investigated if PKD2 also interacts with HDAC5. We confirmed this interaction and mapped the binding of PKD2 to HDAC5 to the same region in HDAC5 that binds to PKD1, suggesting that *TRIM63* expression is redundantly controlled. We also show that PKD3, which is normally not expressed in unstressed myocytes (Li et al., 2011), elicits comparable effects toward the control of *TRIM63* expression by class IIa HDACs. This state-of-affair might especially be important during stress situations where PKD3 was shown to be strongly increased (Li et al., 2011). Our data are in line with previous reports showing that PKD3 can substitute for PKD1 as an HDAC5 kinase in non-muscle cells (Matthews et al., 2006). In summary, our data indicate that a diverse and multilevel pathway regulates muscle atrophy.

Subcellular localization of class IIa HDACs is strongly controlled by several protein kinases, which are not restricted to the protein kinase D family but also involve CamK I, CamK IV (also shown here), MARK2, and others (Chang et al., 2005). Also, some kinases selectively target specific class IIa HDACs, i.e., CamK II specifically targets HDAC4 (Backs et al., 2006). These data, together with our findings and the

known regulation of TFEB and TFE3 activity by posttranslational modification (Puertollano et al., 2018), indicate that TFEB- and TFE3-mediated transcriptional activity has a much higher level of complexity that could not be addressed here and warrants further studies.

Previous studies showed that TFEB (Settembre et al., 2012) and TFE3 (Martina et al., 2014) are predominantly localized in the cytoplasm of HEK-293T and ARPE-19 cells, respectively, and that their phosphorylation status and cytosolic-to-nuclear shuttling regulates TFEB and TFE3 activity. The kinases mammalian target of rapamycin complex 1 (mTORC1) and ERK are important in that regard (Martina et al., 2012, 2014; Settembre et al., 2012). In the presence of nutrients, mTORC1 phosphorylates TFEB and TFE3, thereby facilitating their binding to 14-3-3 chaperone proteins and mediating their retention in the cytoplasm. Conversely, reduced mTORC1 activity increases TFEB (Settembre et al., 2012) and TFE3 (Martina et al., 2014) shuttling into the nucleus. In contrast, in line with our previous report (Du Bois et al., 2015), we observed that TFEB and TFE3 were mainly localized to the nucleus of COS-7 cells when overexpressed. If these observations are attributable to differences in the cell type, culturing conditions, differentiation status, or transfection warrants further investigation. However, our observation that TFEB and TFE3 are localized to the nucleus is supported by other findings showing that 20–30% of TFEB is contained in the nucleus of several cell lines, such as patient-derived fibroblasts (Song et al., 2013), HeLa cells (Settembre et al., 2011), ARPE-19 cells (Martina et al., 2012), and mouse embryonic fibroblasts (Sardiello et al., 2009). Because we hypothesized that HDAC4, HDAC5, and HDAC7 regulate the activity of TFEB and TFE3 at the *TRIM63* promoter, as reported for their inhibitory effects on MEF2 target genes (Lu et al., 2000a,b; McKinsey et al., 2000; Zhang et al., 2002), we used overexpression as a model system despite differences in subcellular localization of the participating proteins. Our data indicate that the activity of TFEB and TFE3 is regulated at least at two different levels, first by regulation of their subcellular localization and second by repression of their activity by class IIa HDACs.

LIMITATIONS

Most of the data shown in our study are based on overexpression experiments. Our results need further evaluation, especially in myocytes, myotubes, and skeletal muscle, and by working with the endogenous components of the signaling pathway described, such as co-staining of endogenous proteins in immunocytochemistry. Nevertheless, our results are in line with previously published work that focused on the regulation of the activity and subcellular localization of class IIa HDACs by PKD both *in vitro* (Zhang et al., 2002; Dequiedt et al., 2005; Backs et al., 2006) and *in vivo* (Fielitz et al., 2008; Kim et al., 2008; Du Bois et al., 2015). Although we have shown that upon coexpression with PKD, HDAC4, HDAC5, and HDAC7 are localized in the cytoplasm, and PKD increased UAS-luciferase activity that depends on 14-3-3 binding, we have not proven that this export was mediated by CRM1. Likewise, to illustrate our findings, we display representative pictures of single nuclei and have not performed biochemical fractionation experiments (nuclear vs. cytosolic extracts) to support the nuclear export of the HDACs upon PKD1 overexpression for a larger subset of cells. Because such experiments have been performed previously, we would like to refer to this work (Harrison et al., 2004; Vega et al., 2004). We have used co-immunoprecipitation experiments to show the interaction of PKD1 and PKD2 with HDAC5, mapped the interacting domains, and showed functional consequences of these interactions. We have also shown that the activity of TFEB and TFE3 on the *TRIM63* reporter is inhibited by class IIa HDACs. However, further studies are needed to prove that these proteins directly interact which each other by using cell-free assays, proximity ligation assays, coimmunostaining, or other techniques.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

CP and BF designed and performed experiments, prepared figures, and prepared the manuscript. YL performed experiments, prepared figures, and provided intellectual input. JR designed experiments and discussed data. FL discussed data, provided intellectual input, and rewrote the manuscript. JF supervised the project, analyzed data, and rewrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.550506/full#supplementary-material

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REVIEW
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Exercise Training as Therapeutic Approach in Cancer Cachexia: A Review of Potential Anti-inflammatory Effect on Muscle Wasting

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Cachexia is a multifactorial inflammatory syndrome with high prevalence in cancer patients. It is characterized by a metabolic chaos culminating in drastic reduction in body weight, mainly due to skeletal muscle and fat depletion. Currently, there is not a standard intervention for cachexia, but it is believed that a dynamic approach should be applied early in the course of the disease to maintain or slow the loss of physical function. The present review sought to explain the different clinical and experimental applications of different models of exercise and their contribution to a better prognosis of the disease. Here the advances in knowledge about the application of physical training in experimental models are elucidated, tests that contribute substantially to elucidate the cellular and biochemical mechanisms of exercise in different ways, as well as clinical trials that present not only the impacts of exercise in front cachexia but also the challenges of its application in clinical practice.

Keywords: muscle wasting, therapeutic exercise, resistance, aerobic, muscle atrophy, systemic inflammation, tumor, neoplasms

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INTRODUCTION

Cachexia (from the Greek *kakos* means "bad," and *hexis* means "condition") is a complex, multifactorial syndrome, with the etiology still unknown; its main characteristic is the involuntary and progressive loss of lean and fat mass (Argiles et al., 2003; Fearon et al., 2012; Blum et al., 2014). In addition, the presence of systemic inflammation, insulin resistance, and anorexia are closely associated with the development of cachexia syndrome, contributing to the increased patient morbidity and debilitation (Inui, 2002).

Cachexia is associated with different types of chronic conditions, such as chronic obstructive pulmonary disease, heart failure, septicemia (Argiles et al., 2010), chronic kidney disease (CKD) (Kir et al., 2016), acquired immunodeficiency syndrome (AIDS) (Tisdale, 2009), and cancer. About 50–80% of cancer patients suffer from cachexia, depending on the type and location of the tumor mass, progressing considerably in advanced stages of the disease (Inui, 2002; Tisdale, 2009). In this sense, the degree of severity and prevalence of cachexia is often associated with the type of

cancer, with gastrointestinal and pancreatic cancers being the most prevalent, reaching more than 85% of cases (Tisdale, 2009). The high prevalence of cachexia in cancer patients contributes to the high morbidity and mortality rate, since cachexia may be directly responsible for 20% of deaths (Vagnildhaug et al., 2017).

Currently, cancer cachexia (CC) is directly associated with systemic inflammation and is characterized as a chronic inflammatory syndrome (Fearon et al., 2006; Evans et al., 2008; Batista et al., 2012b; Argiles et al., 2014) elicited by the interaction between the tumor and the host, which triggers a subsequent immune response. As a result, there is an increased infiltration of cells of the immune system in affected tissues, especially in adipose tissue (Henriques, 2020) and skeletal muscle (Anoveros-Barrera et al., 2019), and consequent release of tumor secreted products, mainly TNFα and IL-6, among others (Tsoli and Robertson, 2013). The release of inflammatory factors alters the homeostasis of various organs and tissues, such as; (1) Hypothalamus, which promotes the reduction of food intake, (2) liver, inducing the production of hepatic acute phase proteins, which repress drug clearance pathways, resulting in higher toxicity of anticancer agents, (3) skeletal muscle, promoting an imbalance between anabolic and catabolic contractile proteins, leading to reduced muscle mass, increased fatigue, and sarcopenia, and (4) brown (TAM) and white (TAB) adipose tissues, increasing lipolysis, reducing adipogenesis (Franco et al., 2017), lipogenesis, and thermogenesis (Tsoli and Robertson, 2013; **Figure 1**).

The maintenance of skeletal muscle mass is reached by a balance between protein synthesis and degradation, which in turn, is mainly regulated by physiological inputs such as the nutritional status and exercise training (Gallagher et al., 2012). Recent studies have focused on the ubiquitin-proteasome pathway, regulation of satellite cells in skeletal muscle, and the importance of related receptors and signaling pathways that are likely to be influenced by tumor-induced systemic inflammation (Fearon, 2011). Lean body mass loss contributes to a progressive weakening in muscle strength and endurance (Gallagher et al., 2012), exercise capacity (England et al., 2012; Jones et al., 2012b) physical activity levels (Wilcock et al., 2008; Dodson et al., 2011), and poor survival (Antoun et al., 2013; Martin et al., 2013). Approaches that aim at increasing increase synthesis or reducing the degradation of muscle proteins, or both, can positively contribute in order to limit or reverse the reduction of muscle mass in patients with CC (Maddocks et al., 2012).

For this purpose, resistance exercise provides a potent stimulator of muscle protein synthesis, and this is mainly confirmed when performed in conjunction with supplementation of branched-chain amino acids (Hakkinen et al., 1998). Although increased muscle proteolysis is also observed immediately after resistance exercise, protein synthesis is stimulated to a greater degree, which provides a positive protein balance resulting in a higher muscle protein content (Schoenfeld, 2010).

It is also noteworthy that the regular practice of exercise, organized in a training program (exercise training), exerts anti-inflammatory effects, which would lead to protection against chronic inflammatory conditions, notably by the reduction of pro-inflammatory cytokines and C reactive protein levels (Petersen and Pedersen, 2005; Fischer, 2006; Lira et al., 2009a; Batista et al., 2010). However, the exact mechanisms of this "beneficial effect," still remain to be elucidated. It is well established that skeletal muscle plays an important role as an immunogenic organ and an important mediator of the anti-inflammatory response (Petersen and Pedersen, 2005; Pedersen et al., 2007; Pedersen and Fischer, 2007). In this scenario, exercise-induced muscle contraction increases interleukin-6 (IL-6) gene expression and secretion by skeletal myocyte, according to the diversity of exercise performance variables (volume, intensity, duration), and an increase in plasma IL-6 levels (Leal et al., 2018). Also, induced by IL-6 (Steensberg et al., 2003a), a subsequent increase in other cytokines as interleukin-10 (IL-10), ILra and soluble tumor necrosis factor I and II receptors (TNF I and II) occurs. The complex secretion of several cytokines is characterized as an "anti-inflammatory effect," a condition observed after an acute session of aerobic exercise (Petersen and Pedersen, 2005).

In fact, exercise training may attenuate the effects of CC via diverse mechanisms, including the modulation of muscle metabolism, insulin sensitivity, and levels of inflammation (Maddocks et al., 2012). Although the many benefits of exercise set a beneficial scenario as an intervention to cachexia symptoms, the lack of studies on the direct evaluation of its effects still raises major questions. Therefore, the present review seeks to elucidate the mechanisms of action of different modalities of exercise training in view of the main symptoms of CC (**Figure 2**). In particular, emphasis will be placed on the overall anti-inflammatory effect of exercise training on muscle wasting, in addition to the possible repercussions on survival during CC.

EXERCISE AS THERAPEUTIC STRATEGY

CC syndrome, as mentioned earlier, is characterized by a progressive weight loss in cancer patients, a condition that interferes with treatment outcomes and directly affects a patient's quality of life (Fearon et al., 2011). In such condition, nowadays, what it is becoming increasingly appreciated at the moment is to find out how the combination of anti-tumor and anti-cachexia therapies, i.e., a synergistic approach would increase the response rates and survival (Laviano et al., 2005).

Currently, there are no standard treatments for cachexia, but recommendations emphasize greater emphasis on the application of a proactive approach in the initial phase of the disease with the intention of maintaining physical fitness or decreasing its rate of decline (Maddocks et al., 2011). In 2011, the consensus published by Fearon et al. (2011) aimed at developing a framework for the definition and classification of CC (syndrome classification according to the stage of evolution and level of involvement), and the most common strategies suitable for treatment at different stages of cachexia (Pre-Cachexia, Cachexia and Refractory Cachexia), as follows: (1) pre-Cachexia stage: patient monitoring and preventive treatment are indicated; (2) cachexia stage: multimodal management according to the presented phenotype, prioritizing contributing factors in order to reverse the picture; (3) refractory cachexia stage: palliative treatment of symptoms,

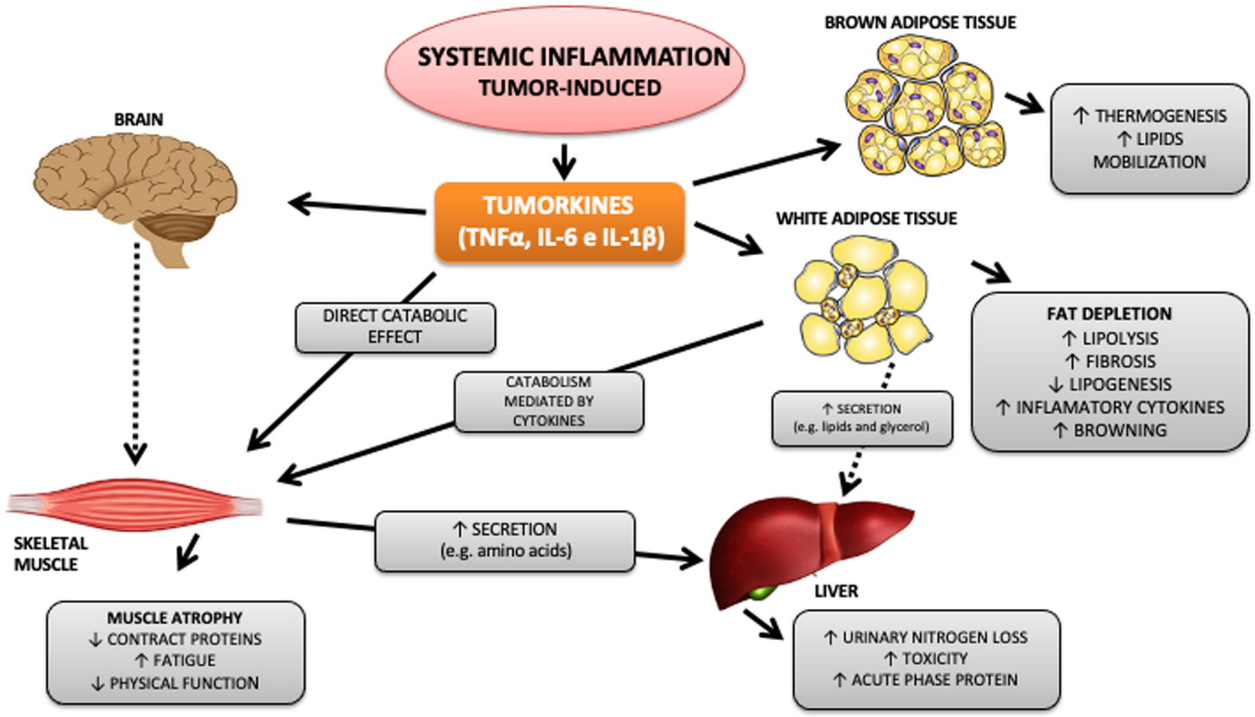

FIGURE 1 | Tumor-induced systemic inflammation and metabolism impact. Among the numerous cytokines released by the tumor, TNFα, IL-6, and IL-1β stand out. These, in turn, are capable of acting on different organs and systems, promoting inflammation, metabolic and immunological changes that directly and indirectly affect essential organs in the pathophysiology of cachexia, such as skeletal striated muscle and white and brown adipose tissue. Continuous arrows refer to mechanisms already described, and dotted arrows refer to mechanisms not yet known.

psychosocial support and ethical discussion about nutritional support are indicated (Fearon et al., 2011).

In the last few years, multimodal treatment has been the most recommended therapeutic approach as an adjuvant intervention in CC. In this sense, the main multimodal components are; (1) multi and interdisciplinary care; (2) treatment of secondary consequences of cachexia; (3) pharmacotherapy directed to inflammatory processes and metabolic alterations; (4) nutritional counseling; (5) physical therapy and exercise training; and (6) social and psychological support (Arends et al., 2017; Naito, 2019). The multimodal treatment has been the most recommended therapeutic approach since CC diagnosed comprises: (1) multi and interdisciplinary care; (2) treatment of secondary consequences of cachexia; (3) pharmacotherapy directed to inflammatory processes and metabolic chaos; (4) nutritional counseling; (5) physical therapy and physical exercise; and (6) social and psychological support (Grande et al., 2015; Arends et al., 2017).

The incorporation of exercise training as a therapeutic strategy should be applied preferentially in the first two stages of the syndrome, pre-cachexia and cachexia, thus attenuates muscle wasting and inflammation. It is noteworthy that in clinical practice, not only the diagnosis, but also the proper classification of the patient between the stages is puzzling, which makes the clinical approach challenging. The lack of proper use of criteria for the diagnosis and staging of cachexia, makes it difficult to determine its prevalence and impact and to assess whether interventions might be adopted in cachectic populations without formal recognition, as the case with exercise studies, particularly in advanced cancer (Grande et al., 2014).

Exercise attenuates the CC effects through different mechanisms, including increased anabolism or maintenance of skeletal muscle homeostasis, improved insulin sensitivity and control of inflammation levels (Grande et al., 2014). Therefore, the implementation of an adequate exercise program combined with an adequate and structured diagnosis according to the treatment stage provides the possibility of reversing protein degradation and stimulates protein synthesis, which guarantees the maintenance of lean mass, ameliorating muscle wasting observed in CC (Gould et al., 2013). Inflammation control is also of the utmost importance in CC as reported in animal and human studies (Seelaender and Batista, 2014).

It has even been proposed that a ratio between pro- and anti-inflammatory cytokines may serve as a marker of CC severity of Argiles et al. (1997). For example, IL-10 (anti)/TNFα (pro) ratio has been adopted as a marker of inflammation level in obese individuals and animals (Jung et al., 2008). The increase in pro-inflammatory state is closely related to the decrease in survival and morbidity in CC (Lira et al., 2009c). Therefore, exercise training, either through the Resistance Training (RT) or endurance training, seems to contribute satisfactorily to the control of inflammation by modulating the balance of the

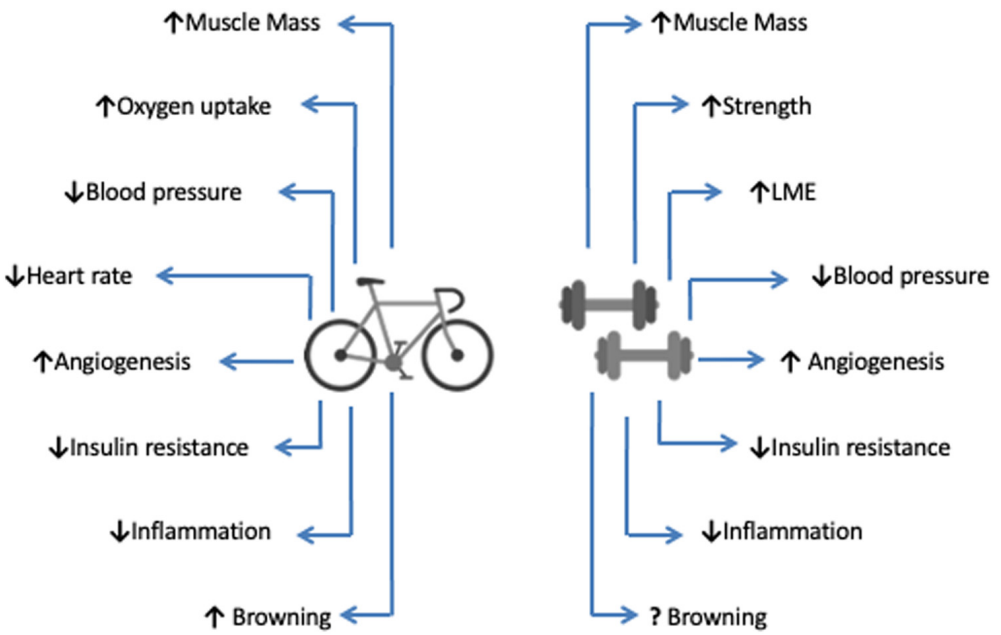

FIGURE 2 | Main physiological effects of aerobic and anaerobic exercise training. Both types of exercise. Both different exercise types promote numerous physiological changes that result in improved physical capacities and health indicators, resulting in increased life expectancy (survival). Aerobic exercise is represented by the bicycle, and the dumbbells describe anaerobic exercise. LME, Local Muscular Endurance.

main pro and anti-inflammatory cytokines (TNFα, IL-6, and IL-10) (Donatto et al., 2013).

Exercise Training as a Modulator of Inflammation

In CC, systemic inflammation may have various origins such as tumor cells themselves as well as activation of immune cells that release cytokines, chemokines, and other inflammatory mediators (Argiles et al., 2014). Although the tumor appears to be primarily responsible for the activation of immune cells, the intestine also plays an important role in this process due to the fact that intestinal barrier dysfunction and bacterial translocation are associated with cancer (Klein et al., 2013). Numerous inflammatory cytokines, such as tumor necrosis factor α (TNFα), IL-6, and interleukin-1 (IL-1), are elevated in cachectic cancer patients and are known to promote catabolism (Argiles et al., 2012a). Although different cancer cells may release distinctive cytokines, the circulating levels from different host tissues appears increased as a response to cancer (Argiles et al., 2009). Multiple humoral factors and cellular signaling pathways appear to be involved in the reduction of cancer-derived muscle mass, which worsens the patient's prognosis. It is believed that systemic inflammation may be the main mediator of this process, and, therefore, unveiling its real origin in cancer patients may contribute to the development of an effective therapy (Zhang et al., 2017).

Among the different types of treatment that aim at reversing or restricting the progression of CC is the use of pharmacological interventions with anti-cachexia agents and the treatment of inflammatory cytokines designed to contain cachexia, especially TNFα (Gould et al., 2013). Among the various non-pharmacological strategies that have been investigated, different modalities of exercise training, such as endurance, resistance training, and combined training appear to act favorably in controlling inflammation, as they are capable of inducing an increase in anti-inflammatory cytokine secretion by adipose tissue causing significant reduction in C-Reactive Protein (CPR) levels (Lira et al., 2009c; Pierce et al., 2009; Galvao et al., 2010).

As already mentioned, multiple inflammatory cytokines such as TNFα, IL-6, and IL-1 are known to promote muscle wasting during CC progression (Zhang et al., 2017). However, it is well characterized that after an exercise session, there is an exponential increase in the levels of IL-6 (more than 100 times) acutely, which is dependent on the configuration of the exercise variables, such as intensity, duration, amount of muscle mass recruited and individual aerobic capacity (Fischer et al., 2003; Steensberg et al., 2003b; Fischer, 2006). It has been observed that administration of IL-6 at levels similar to those observed in the plasma of subjects following long-term and high intensity exercise is capable of promoting significant reductions in TNFα levels (Starkie et al., 2003). Such evidence is corroborated by studies that evaluate the rates of pro-inflammatory cytokines in animals with induced cancer undergoing exercise training. Interesting, High-Intensity Interval Training (HIIT) efficiently increases survival rate and reduces tumor mass growth in tumor-bearing mice (Alves et al., 2018). Similar data can be found in a study that evaluated the effects of voluntary running against tumor progression and survival of animals with different tumor types. Also, a positive correlation was observed between increased cytokine expression (especially IL-6) by tumor cells and activation and redistribution

of Natural Killer cells, which in turn is directly responsible for suppressing tumor growth by up to 60% (Pedersen et al., 2016).

Adipose Tissue (AT) is deeply affected during CC and might play an important role in systemic inflammation (Batista et al., 2012a). For instance, epididymal AT presents an early impairment of lipid synthesis and storage, which is not observed in the mesenteric area, more resistant to the "fat reduction effect" of CC more than any other depot. In addition, an increase in the size of adipocytes and a consequent increase in the production of pro-inflammatory proteins, such as tumor necrosis factor alpha (TNFα) and membrane receptors (TNFR1 and TNFR2) (Figueras et al., 2005) are reported. On the other hand, endurance training is able to induce a higher production of IL-10 from AT, an important anti-inflammatory cytokine, which contributes to modulate TNFα secretion and control inflammation (Lira et al., 2009b).

The anti-inflammatory effect of exercise is more evident in certain pathological conditions, such as atherosclerosis, type II diabetes mellitus, obesity and chronic heart failure (CHF), conditions characterized as low-intensity chronic systemic inflammation and by a twofold to a threefold systemic increase in pro-inflammatory cytokine and C-reactive protein levels (Fischer et al., 2003).

Inflammatory cytokine levels when measured in the AT of animals with induced CC and submitted to RT is deeply modulated. The levels of IL-10, TNFα, and IL-6, and the ratio between IL-10 and TNFα are systemically up-regulated, but the levels of IL-6 in AT are down-regulated. These findings were followed by a reduction in tumor volume and the preservation of muscle mass (Donatto et al., 2013). Endurance training also improves the IL-10/TNFα ratio and induces a reduction in monocytes infiltration into different visceral adipose tissue depots (mesenteric and retroperitoneal) of tumor-bearing rats (Lira et al., 2012; **Table 1**).

Few studies in clinical practice, to date, have investigated the systemic effects of training on the inflammatory profile during CC (Lira et al., 2012; Das Neves et al., 2016). With this limited range of information, it becomes complex to extrapolate outcomes to cancer patients, especially at an advanced stage. To this end, further studies to explore the effects of training on both systemic and local inflammation markers are required.

Exercise Training and Muscle Atrophy in Cancer Cachexia

Muscle wasting is one of hallmarks of CC (Fearon et al., 2011; Fearon, 2011; Bedard et al., 2015). During CC, muscle protein breakdown occurs disproportionately compared to other tissues, causing widespread weakness and debilitation. Once respiratory muscles are affected asphyxiation and death may take place (Cai et al., 2004).

Three systems are described for acting on protein degradation in skeletal muscle: the lysosomal system; the activated calcium system; and the ubiquitin-proteasome system (UPS), which degrades most cellular proteins (Tisdale, 2009). The latter is an ATP dependent process of labeling protein substrates with ubiquitin molecules, mediated by Ubiquitin E3 Ligases (Bilodeau et al., 2016).

It has been observed in different studies that moderate-intensity running is able to stimulate muscle hypertrophy, reduce autophagy markers, increase mitochondrial activity and reduce gene expression of Murf and Atrogin-1 Ubiquitin ligases in the muscle of BALB/c mice with C26-induced cachexia (Pigna et al., 2016; Ballaro et al., 2019). However, when combined with climbing (combined training) the total mass reduction is not inhibited, but only the activity of autophagy markers without altering the UPS system (Ranjbar et al., 2019; **Table 1**).

Different mechanisms are involved in reducing muscle mass during CC. Several hormones, cytokines and tumor-derived factors have been shown to influence protein balance in normal and disease situations through various intracellular signal transduction systems (Zhou et al., 2010). While the IGF1/PI3K/AKT pathway is related to muscle protein synthesis, an opposite pathway mediated by activation of FOXO transcription factors as well as the NF-κB pathway in conjunction with Smad transcription factors promote protein breakdown (Glass, 2005).

Myostatin, a member of the TGFβ growth factor superfamily, is considered a negative regulator of skeletal muscle mass under many conditions of muscle loss (Smith and Lin, 2013) detected at high levels during the embryonic and postnatal developmental stages (Argiles et al., 2012b). Myostatin deletion increases muscle regeneration through satellite cell activation and self-renewal, thus promoting postnatal muscle growth and repair (Mccroskery et al., 2005). Several different types of myostatin pathway inhibitors, including anti-myostatin antibodies and their receptor (ActRIIB), are under clinical development. Preliminary results point to the preservation of muscle mass in patients with muscular dystrophy and cancer (Lee et al., 2005; Zhou et al., 2010).

In addition, myostatin works to inhibit muscle growth and regeneration by regulating MyoD (myogenic differentiation factor) expression, resulting in reduced muscle mass. MyoD is a direct target of the NF-κB transcription factor activation cascade that is activated during cachexia by TNFα (Guttridge et al., 2000). Considering NF-κB as a potent inhibitor of MyoD expression, there is a possibility that myostatin could signal via NF-κB to regulate MyoD expression (Mcfarlane et al., 2006; Coletti et al., 2016).

The mechanism involved in muscle protein degradation during LLC-induced cachexia has been addressed by Zhang et al. (2017). Co-culture of C2C12 myotubes in LLC cell-treated medium, rapid activated catabolic response in a TLR4-dependent manner, including activation of the p38 MAPK-C/EBPβ signaling pathway, as well as ubiquitin-proteasome and autophagy pathways resulting in myotube atrophy. In contrast, TLR4 knockout animals with induced CC showed muscle mass preservation and lower levels of TNFα and IL-6 compared to wild-type animals. Taken together, the data set suggests that TLR4 is the central mediator of muscle atrophy observed in CC and also an important therapeutic target.

Toll-like receptors (TLRs) are highly conserved transmembrane proteins that play an important role in the detection and recognition of microbial pathogens (Lu et al., 2008). TLR4 acts as a lipopolysaccharide (LPS) receptor, and

TABLE 1 | Studies published in the last 10 years with experimental rat and mouse models.

Author	Animal/culture		Cachexia markers	Exercise training protocol			Main results
	Species/ lineage	Induction method		Type	Protocol	Volume/ intensity	
Ballaro et al. (2019)	Female BALB/c mice	5×10^5, C26 tumor cells	↓ Total body mass ↓ Muscle mass ↓ Adipose tissue mass	Aerobic training	Moderate exercise on treadmill	Running speed by 11 m/min for 45 min	• Exercise-induced a decrease in muscle autophagy markers • Exercise improves mitochondrial mass and activity in skeletal muscle • Exercise reduces muscle gene expression of MuRF1 and Atrogin-1
Ranjbar et al. (2019)	Balb/c mice	5×10^5 C26 carcinoma cells	↓Body weight ↓Muscle weight ↓Functional capacity	Combined training	1° Resistance Training (ladder protocol) and; 2° aerobic training (motorized Wheel), on the same day	4 days/week, 4 weeks before and 11 days after tumor implantation RT - 3 sets of 2 repetitions at 50% of animal body weight AT - 25 min. 5–9 m/min speed	• Exercise does not restore body weight loss • Exercise does not prevent gastrocnemius loss but increase tibialis anterior weight • Training was able to reduce autophagy markers in muscle • Exercise does not decrease atrophy markers
Alves et al. (2018)	C57BL/6	10^6 LLC cells	No control group was presented to indicate presence of cachexia	High-intensity interval training (HIIT)	Treadmill running	Each session consisted of 5 intervals of 3 min running at 18 m min^{-1} followed by 4 min running at 25 m min^{-1}	• Decrease on tumor size in tumor trained group • Increase on PDL1 and VEGF expression in tumor • Increase of survival
Das Neves et al. (2016)	Wistar rats	10^6, Walker 256 tumor cells injected in the bone marrow	Muscle atrophy, inflammatory markers (TNFα)	Resistance training (RT)	Hind limb (i.e., "squat-like" movement). Started after tumor injection with daily sessions for 15 days	1–3 sets; 10–15 rep; 65% 1RM	• Cancer-induced not effect on total body mass • Muscle atrophy with no changes in RT group • No difference in atrophy markers • Loss of strength associated with decrease on survival
Khamoui et al. (2016)	Female Balb/c mice	5.0×10^5, C26 tumor cells	↓ Total body mass, muscle and fat mass ↓ Physical function	Resistance and aerobic training	Training started 8 weeks before tumor injection and during 3 weeks after tumor injection RT was performed on a climb ladder AT in a motorized wheels	• RT—3 days per week, 5 sets of 3 repetitions with progressive loads • AT—60 min, 5 days per week. Speed between 5–6.5 m/min	• RT and AT not restore total and lean or fat mass in C26 group • ↑ In IGF-1 gene expression in muscle of RT-C26 and AT-C26 • ↑ In Myogenin gene expression in muscle of RT-C26 group • No differences in physical function in trained groups
Pigna et al. (2016)	Female BALB/c mice	Grafting of 0.5 mm^3 fragment of colon carcinoma (C26)	↓ Total body mass ↓ Muscle mass	Aerobic training	Wheel-running activity	Running for 20:59 ± 4:30 (h min) a day at Speed by 2.1 ± 0.1 (km/h)	• Increase in life span in wheel running C26-bearing mice • Exercise-induced muscle hypertrophy in C26-bearing mice • Exercise-induced a decrease in muscle autophagy markers • Exercise reduces muscle gene expression of MuRF1 and Atrogin-1
Pedersen et al. (2016)	Female C57BL/6 and NMRI-Foxn1nu mice	2×10^5 B16F10 melanoma cells 2×10^5 LLC tumor cells	↓Body weight ↑Inflammation	Running wheels	Voluntary running, started 4 weeks prior to tumor cell inoculation	Only total running distance was evaluated	• Running decrease tumor growth • Upregulation of pathways associated with immune function • Levels of epinephrine and IL-6 are related to tumor growth through mobilization of NK cells

(Continued)

TABLE 1 | Continued

Author	Animal/culture		Cachexia markers	Exercise training protocol			Main results
	Species/ lineage	Induction method		Type	Protocol	Volume/intensity	
Pin et al. (2015)	Balb/C or C57BL/6	5×10^5 Colon26 or LLC cells	↓ Muscle mass ↓ Physical function ↓Hematocrit content	Aerobic training	Treadmill Running, 5 days/week, 14 or 28 days after C26 or LLC implantation, respectively	Mice ran for 45 min at the speed of 14 m/min	• In the C26 hosts, acute exercise does not prevent muscle wasting • LLC hosts are responsive to exercise and their treatment with the EX-EPO combination prevents the loss of muscle strength • LLC EX-EPO group increases muscle oxidative capacity
Donatto et al. (2013)	Wistar rats	Walker 256, 3×10^7 tumor cells	↓ Total body mass ↓ Muscle mass inflammatory markers	Resistance training	8 weeks of resistance training, with climbing sessions	Training sessions were 3–5 ladder climbs with 75, 90, and 100% of the rat's previous maximal carrying capacity	• RT increased by 9% body weight gain in TB group • LDL-c levels were decreased with RT in TB group • HDL-c levels were increased with RT in TB group • IL-10/TNFα ratio was higher with RT in TB group • RT attenuate the protein content of IL-6
Lira et al. (2012)	Wistar rats	Walker-256 carcinosarcoma (2×10^7 cells/rat)	↓Body weight	Aerobic training	8 weeks of treadmill running	15–60 min at 10 m/min. Intensity maintained between 60 and 65% VO2 max	• Training groups showed decrease on adipose tissue content; • Decrease on tumor size in tumor trained group • Exercise tumor group showed decrease of IL-6 MEAT content • Exercise tumor group showed decrease on IL-10/TNFα ratio in MEAT group • Decrease of macrophages infiltration on adipose tissue with training
Penna et al. (2011)	C57BL/6 mice	5×10^5 LLC cells	↓ Muscle mass ↓ Adipose tissue mass	Aerobic training	Treadmill running	14 m/min, 45 min, 5 days/week	• Exercise + EPA treatment attenuate muscle wasting • Exercise + EPA improve muscle strength • Exercise + EPA reduce tumor weight

TNFα, Tumor Necrosis Factor-Alpha; RM, Maximal Repetition; RT, Resistance Training; AT, Aerobic Training; IGF-1, Insulin-Like Growth Factor-1; C26, Colon-26 Adenocarcinoma; MURF-1, Muscle RING-Finger Protein-1 (Murf1); ATROGIN-1, Muscle-Specific Ubiquitin Ligase; EPA, Eicosatetraenoic Acid; LLC, Lewis Lung Carcinoma; TB, Tumor Bearing Group; LDL, Low Density Lipoproteins; HDL, Hight Density Lipoproteins; IL-6, Interleukin Type 6; NK, Natural Killer Cells; PDL-1, Programmed Death-Ligand 1; VEGF, Vascular Endothelial Growth Factor.

associates with myeloid differentiation protein 2 (MD2) to form a complex to interact with LPS (Henriques et al., 2018). Both acute and chronic endurance aerobic exercise are cited for decreasing TLRs expression on the monocyte cell surface, also decreasing inflammatory cytokine production and TLR4 cell surface expression in monocytes. These effects are related to post-exercise immunosuppression and are responsible for increasing athletes' susceptibility to infection (Gleeson et al., 2006).

Few studies are investigating the impact of exercise training on Toll-like receptors. It was shown that 1.5 h of cycling (∼65% VO2 max) in heat (34°C) acts on TLR expression and function *in vivo* (healthy men), followed by a reduction in post exercise (immediately after the end of session) TLR1, TLR2 and TLR4 expression and after 2 h of recovery (Lancaster et al., 2005). The acute effects of eccentric resistance training on the TLR4 signaling pathway were evaluated in men who underwent two eccentric RT sessions at 6 weeks intervals, with 1 group continuing the training and while the other remained at rest. Acute eccentric RT increased TLR4-mediated NF-κB and MAPK activation and TNFα levels in human peripheral blood mononuclear cells, suggesting a pro-inflammatory response. However, the 6 weeks RT group reduced TLR4-mediated activation of the pro-inflammatory response via independent and MyD88-dependent pathways (Fernandez-Gonzalo et al., 2012).

NF-κB has been considered a pro-inflammatory signaling pathway activated by pro-inflammatory cytokines such as IL-1

and TNFα (Lawrence, 2009). NF-κB in the muscle is activated by disuse or sepsis and plays an important role in the pathogenesis of these conditions, however, there are other alternative intracellular pathways, such as caspases and JNK/AP-1, which are also capable of being activated by cytokines (Cai et al., 2004). NF-κB blockade is able to inhibit protein catabolism in C2C12 myotubes (Li and Reid, 2000). However, although the mechanisms of action of NF-κB are established in innate immunity, inflammation and apoptosis, the role of this transcription factor in the muscle is not yet fully understood.

As already mentioned, intense exercise acutely promotes a significant inflammatory response. In RT practitioners, after an intense training session, IκBα muscle protein levels decreases (a member of a family of NF-κB inhibiting cellular proteins), while p-NF-κB (p65) protein levels increase by 2 h after exercise and return to near-basal levels within 4 h of exercise. In addition, it has been observed that both the circulating levels and the MCP-1, IL-6 and IL-8 mRNA are up-regulated significantly 2 h after exercise. These findings indicate that intense resistance exercise transiently activates NF-kB signaling in human skeletal muscle during the first hours after exercise (Vella et al., 2012). Another study has investigated the effects of endurance exercise to exhaustion in trained rats finding that RT increases p50, IκBα, and phosphor-IKK kinases content in muscles. Moreover, temporal analysis indicates that corroborating the previous study, higher levels of NF-κB ligands were observed 2 h after exercise, while p65 reached maximum levels between 2 and 4 h. The authors suggest a link between increased muscle activation of NF-κB to exercise redox factor (Ji et al., 2004).

In summary, systemic inflammation is the main cause of muscle atrophy and fatigue in CC, clinical marks also described in the aging process, AIDS, chronic heart failure, COPD and also in CC (Li et al., 2008). On the other hand, RT present itself as an alternative type of training capable of modulating CC independently of drugs, mainly due to its ability to promote an anti-inflammatory scenario.

EXERCISE AND TUMORAL PROGRESSION

The first published paper on the effects of exercise on tumor growth was conducted in 1944 (H. P. Rusch and Kline, 1944). The influence of TR on the rate of tumor growth in mice transplanted with fibrosarcoma was investigated; in this case, the animals preserved weight loss and the rate of tumor growth was also lower than that observed in the control group. As a consequence, a precedent was opened for numerous reports seeking to elucidate the mechanisms involved.

Another study, published in 1962, sought to evaluate the effects of combined high intensity running-swimming training, both performed to fatigue, on Wistar rats inoculated with Walker 256 tumor. The results showed that tumor weight and size in control animals were greater compared to the trained groups. There were even cases of complete tumor regression in the exercised animals (Hoffman et al., 1962). In another trial performed in the same work one group received an injection of an inhibitory substance called (F-Substance) produced by fatigued muscles (muscles were suspended with a 0.85% NaCl bath and electrically stimulated to "fatigue"). F-substance simulate the effects of exercise and assessed whether it would inhibit tumor growth in a similar way to RT. The results were compatible with those found in the trained group; however, authors did not investigate the components of the substance or its mechanism of action (Hoffman et al., 1962).

Nonetheless, two recent studies using Walker 256 tumor-induced cachexia that performed RT (climbing training) present divergent data on tumor volume. One showed that the trained animals did not undergo any changes in tumor mass compared to their matched control; however, muscle mass was preserved and inflammatory markers such as TNFα and IL-6 were reduced in the plasma of animals submitted to RT (Donatto et al., 2013). The other study showed a 10% reduction in tumor volume ($p < 0.05$) (Bacurau et al., 2007), and lower circulating levels of inflammatory markers. However, a study evaluating the effects of two different types of training, aerobic and RT training, on induced cachexia in Balb/c mice found that the animals that practiced RT presented a tumor volume increase of about 30% (Khamoui et al., 2016).

A study evaluating the effects of voluntary running on animals with CC induced by different tumor types found a positive regulation of both pro- and anti-inflammatory cytokines (IL-6, IL-1β, TNFα, iNOS), intense mobilization and trafficking of NK cells (natural killer) and lower incidence and reduction of tumor volume by up to 60% (Pedersen et al., 2016). Also, CD-68 expression (expressed in monocytes, used as a marker for macrophages), NKp46 (metastatic control mediator), NKG2D (NK cell marker) and FoxP3 (T-cell negative regulator) were increased. The author linked this positive regulation of immune system-related pathways with greater activation and infiltration of NK cells into the tumor, which suppressed its growth. In the same study, an increase in inflammatory markers such as IL-1β, IL-6, iNOS, and TNFα was also observed in the tumor of voluntary runners (Bay et al., 2017).

Tumor progression in C57bl/6 mice with LLC-induced cachexia and practicing high intensity interval training through treadmill running was analyzed in a recently published work. It has been observed not only a reduction in tumor volume, but also a higher expression of CD274 (PD-L1), a transmembrane protein responsible for suppressing the immune response through T cell presence in the tumor of trained animals. VEGFα, an important marker of vascularization, was also increased with training in combination with a tendency to increase IL-6 and TNFα expression (Christiano et al., 2018; **Table 1**).

Hypoxia and lack of blood supply promote an aggressive cancer phenotype, which contributes to the inefficiency of systemic therapy (Shannon et al., 2003). In contrast, vascular normalization, for example, stimulation of vascular endothelial growth factor (VEGF), improves chemotherapy response by improving oxygenation and consequently increased access of drugs to the tumor. Aerobic exercise stimulates a more "normalized" tumor microenvironment by improving intratumoral perfusion/vascularization, as demonstrated in animal models of breast and prostate tumors (Ruiz-Casado et al., 2017).

The mechanisms postulated for the potential effects of exercise on cancer progression include metabolic modulation through evaluation of host glucose-insulin homeostasis and modulation of sex hormones (testosterone), improvements in immune surveillance, and reduction of systemic inflammation and oxidative damage (Jones et al., 2012a). The comprehension of the mechanisms elicited by RT involved in tumor growth control are still at an early stage of development and understanding since the heterogeneous nature of the tumors and the biological variation of the host are challenging variables (Claudia et al., 2017).

PHYSICAL EXERCISE AND SURVIVAL

Studies evaluating the effects of exercise training on the survival of patients diagnosed with CC are still scarce. A search of PubMed's databases was performed by crossing the terms: survival, cancer and exercise, selecting only clinical studies conducted in the last 10 years. With these descriptors a total of 178 papers were found. However, when the search was performed with the replacement of the descriptor "cancer" by "cancer cachexia," with the same parameters as before, only three studies were found, available in **Table 2**. In addition, a recent Cochrane review showed that there are no randomized control studies that have addressed exercise interventions in CC (Grande et al., 2014). Notwithstanding, only one of these studies (Solheim et al., 2017) had as inclusion criteria the diagnosis of cachexia according to the international consensus diagnosis and classification of cachexia (Fearon et al., 2011).

The unique study investigated the effect of multimodal treatment consisting of anti-inflammatory pharmacological treatment, use of EPA-enriched hyperproteic and hyperproteic supplement (ProSure© Abbott), nutritional counseling, and exercise prescription to be performed outside the hospital setting in patients with liver and pancreas cancer during anti-cancer treatment. This was a randomized phase II study, and the results were similar between groups for muscle mass, grip strength, inflammatory, and fatigue parameters. The authors linked these factors to poor treatment adherence (final $n = 25$) (Solheim et al., 2017). Besides, it should be noted that several studies are showing a positive effect of exercise training on the survival of cancer patients, without the presence of cachexia (Holmes et al., 2005; Liu et al., 2006; Holick et al., 2008; Irwin et al., 2008).

Besides, three different experimental model studies using rats or mice, published in the last 10 years, were found. Those experimental models have evaluated the effect of different exercise protocols in the presence of induced cachexia, as available in **Table 1**. Wistar rats with Walker 256 tumor-induced cachexia submitted to RT (climbing), presented a reduction in survival compared to sedentary tumor animals (Das Neves et al., 2016). The tumor was inoculated into the brown bone marrow and induced a severe reduction in muscle content and physical function that was positively correlated with poor survival. In another study, voluntary running was used as an intervention in C26 tumor-inoculated female BALB/c mice; the presence of running wheels increased the survival of tumor-bearing mice, however, this positive affect was not attributed to environmental enrichment (Pigna et al., 2016). Finally, when a High Intensive Interval Training (HIIT) treadmill training was performed by C57bl/6 Tumor bearing mice, animals presented an increased survival, and reduced tumor volume (Christiano et al., 2018).

The real contribution of exercise and its impact on the life expectancy of patients diagnosed with CC remain an obscure field. However, the numerous local and systemic benefits of

TABLE 2 | Studies published in the last 10 years with cancer patients.

Author	Subjects	Cachexia markers	Exercise training protocol			Main results
			Type	Protocol	Volume/intensity	
Grote et al. (2018)	Oncologic patients with diagnosed cachexia	↓Body weight ↓Functional capacity	Progressive resistance training	13 training sessions, 3 times weekly for 30 min	3 exercises for major muscle groups with 8–12 repetition maximum	• Improvement of weight loading • Improvement in general fatigue and quality of life in the intervention group
Solheim et al. (2017)	Oncologic patients with diagnosed cachexia	Body mass index < 30 kg/m^2; and $< 20\%$ weight loss in the previous 6 months	Multimodal treatment: anti-inflammatory drug; Eicosapentaenoic Acid (EPA); Nutritional counseling; and Exercise program including home-based aerobic and resistance training	Aerobic exercise as patients' choice and resistance training	AT for 30 min a Day, 2 times a week; RT consisted in six individualized exercises, three times weekly for about 20 min	• No statistically significant effect on physical activity or muscle mass • Survival was similar between the groups
Rogers et al. (2011)	Oncologic patients with diagnosed cachexia	↓Body weight ↓Functional capacity	Resistance Training + EPA or Cox-2 Inhibitor	20 weeks of RT. 5-10 min warm up, followed by the exercise prescription, and a 5 min cool-down	Not mentioned	• Increase in grip strength • Increase in body weight • Improved levels of fatigue • Decreasing CRP and interleukin-6

EPA, eicosapentaenoic acid; COX-2, cardiomyocyte cyclooxygenase-2; CRP, C-reactive protein.

exercise are already being clarified (Ruiz-Casado et al., 2017). Exercise is recognized to trigger the formation and secretion of several muscle cytokines, including IL-6, which increases insulin sensitivity and reduces the production of pro-inflammatory cytokines (Grande et al., 2014). Chronically, exercise has a global anti-inflammatory effect, which has been observed in healthy people and early stage cancer patients (Betof et al., 2013). Such an effect would be beneficial in CC as levels of systemic inflammation are associated with weight reduction, exercise capacity and survival (Laird et al., 2013).

PROSPECTS AND LIMITATIONS ON THE USE OF EXERCISE IN CACHEXIA

Exercise training comprises a critical strategy to be used as therapy CC. The exercise effect is related to supporting protein synthesis and muscle growth, resulting in muscle strengthening and improved physical performance. Besides, exercise induces an anti-inflammatory response, potentially abrogating catabolic effects, the primary marker of muscle wasting. In this context, the most suitable modality is based on resistance exercises. **Figure 3** illustrates a synthesis of all the events promoted by the different types of exercise against CC up to date mentioned in the literature. Protein synthesis is stimulated to a higher extent than the proteolysis observed after the acute exercise session, which contributes to the protein balance. However, as mentioned above, inflammation control (reduction) is a crucial need for patients with CC. In this scenario, the pivotal role of exercise to stimulate the release of a plethora of cytokines, mainly by the skeletal muscle, so-called myokines, must be considered. The main responses observed as a result of the action of myokines are their ability to improve glucose uptake by muscles, muscle lipolysis and fat oxidation, and thus, mobilize energy reserves. In the case of IL-6, which is the predominant cytokine produced in response to exercise, it is indicated that chronically, after exercise, it is responsible for increasing insulin sensitivity and reducing pro-inflammatory cytokine production. Endurance exercise, in this case, is indicated as a safe and efficient alternative for the control of inflammation observed in patients with CC, considering that the cardiovascular condition is compatible with the prescribed exercise load. Taken together, a proper training prescription should contain the combination of strength and endurance work to meet therapeutic needs. The challenges regarding cancer patients' adherence to the exercise programs are primarily related to their fatigue tolerance to

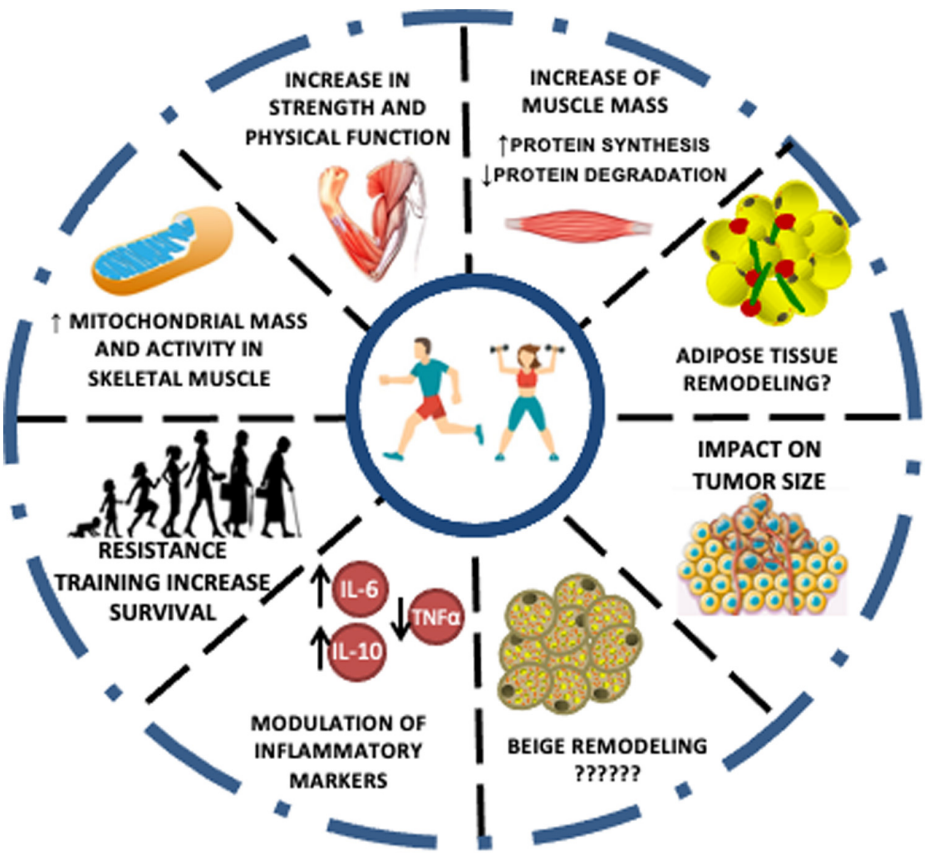

FIGURE 3 | Effects of exercise training in cachexia markers. The effects of different physical training types on cachexia symptoms and markers are evident in the literature; however, in a majority way through tests on experimental models. The changes promoted in skeletal muscle and inflammation control are highlighted as the leading promoters of a better prognosis and increased life expectancy elevated by exercise training.

exercise. Patients with lower baseline fatigue scores and greater adherence to the intervention had the greatest improvements. It is worth noting that patients who previously had lower cancer-related fatigue scores have a greater ability to tolerate exercise and therefore better outcomes (Puetz and Herring, 2012). However, a study of patients undergoing exercise during chemotherapy treatment indicated that as exercise exposure increased the intensity of cancer-related fatigue decreased at all basal levels of fatigue. The authors also highlighted that despite the advanced stage of the tumor and the costly treatment, adherence to the intervention was excellent. Progressive resistance training in patients with head and neck cancer cachexia during radiotherapy proved to be safe and its effects are beneficial in the face of fatigue and quality of life (Grote et al., 2018). Another study indicated that progressive resistance training in cancer patients with cachexia is feasible. The training proved to be well tolerated and safe (Grote et al., 2018; **Table 2**).

The role of exercise training in CC needs further elucidation in the coming years. Studies of exercise interventions in the field are few in number and most concentrated in animal models. In cachexia patients, there are just a few reports of small studies from conference proceedings as well as ongoing/planned studies. It demands additional randomized trials to investigate exercise training either alone or in combination with pharmacologic treatment. Different variables were indicated as determinants for adherence to the exercise program, such as location of the training center, disease stage, aerobic conditioning, and depression (Schwartz et al., 2001). Given this potential, the major challenge in the applicability of an exercise training program in cancer patients comprises a number of variables such as: (1) prescription aligned according to the proper diagnosis of the cachexia stage in which the patient is; (2) patient motivation for adherence and permanence in the program; and (3) adequate and constant control of training variables so as not to exceed the patient's physical condition.

AUTHOR CONTRIBUTIONS

MB, LL, and ML conceived the review and wrote the manuscript. LL, ML, and SP analyzed the data. All authors contributed to the article and approved the submitted version.

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Invalidation of the Transcriptional Modulator of Lipid Metabolism PPARβ/δ in T Cells Prevents Age-Related Alteration of Body Composition and Loss of Endurance Capacity

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Anti-inflammatory regulatory T cells (Tregs) are the most metabolically flexible $CD4^+$ T cells by using both glycolysis and fatty acid oxidation (FAO) which allow them to migrate in tissues. With aging, Tregs accumulate in secondary lymphoid organs and are involved in impairment of skeletal muscle (SKM) regeneration and mass maintenance. In this study, we showed that a deletion of a FAO modulator, peroxisome proliferator-activated receptor beta/delta (PPARβ/δ), specifically in T cells (KO-T PPARβ/δ), increased the number of $CD4^+$ T cells at day 2 following a cardiotoxin-induced SKM regeneration. Older KO-T PPARβ/δ mice maintained a Tregs prevalence in lymph nodes similar to young mice. Surprisingly, KO-T PPARβ/δ mice were protected from the effects of age on lean and fat mass and endurance capacity. Our results lead us to propose an original potential role of T cell metabolism in the effects of aging on the maintenance of body composition and endurance capacity.

Keywords: regulatory T cells, skeletal muscle, aging, immunometabolism, physical capacity

INTRODUCTION

Agonists of the peroxisome proliferator activated receptor beta/delta (PPARβ/δ) have been studied this last decade as "exercise-mimetics" that are susceptible to be therapies for metabolic diseases by increasing skeletal muscle (SKM) fatty acid metabolism (Wall et al., 2016; Fan et al., 2017). The expression level of PPARβ/δ is one of the determinants of its activity (Rousseau et al., 2016) and of its physiological role in the regulation of inflammation (Neels and Grimaldi, 2014). Although PPARβ/δ is highly expressed in SKM, it is broadly expressed in other tissues including lymphoid organs (Girroir et al., 2008) where its activity increased after administration of its specific agonist GW0742 (Le Garf et al., 2019). A critical role of PPARβ/δ in T cell functions has been suggested by studies

using whole body PPARβ/δ-deficient mice (Dunn et al., 2010; Kanakasabai et al., 2011; Zhao et al., 2018). However, these studies did not allow determining whether the effects observed on immune dysregulations were directly induced by specific PPARβ/δ absence in T cells. By treating mice with GW0742 or by using a mouse model specifically overexpressing PPARβ/δ in T cells, we showed a drastic decrease in alpha/beta T cells that was accompanied by a characterized thymus involution (Mothe-Satney et al., 2016). This might reflect an adaptation of the energetic metabolic cost of thymus function and resemble, in some aspects, to changes observed with aging (Sato et al., 2017). Immune aging is associated with a decline in T cell activation and proliferation involving alteration of signaling pathways in T cells such as nuclear factor kappa B (NF-κB) (Bektas et al., 2013, 2017) which activity can be regulated by PPARβ/δ (Schnegg et al., 2012). In addition, alteration of lipid metabolism, that determines lipid rafts and fatty acid oxidation (FAO), also contributes to the decline in T cell functions with age (Larbi et al., 2004) and their ability to survive (Yanes et al., 2019). Whether this aging effect involves an increased activation of PPARβ/δ is unknown but overexpression/high activity of PPARβ/δ in T cells in healthy animals could be potentially deleterious for the maintenance of a normal immune function.

We have previously shown that $CD4^+$ T cells are able to increase their FAO after *in vitro* treatment with GW0742 (Mothe-Satney et al., 2016). The delineation of the different utilization of metabolic pathways by distinct subsets of T cells (Michalek et al., 2011; Newton et al., 2016) has led to the exciting possibility that PPARβ/δ activation could increase the presence of subsets which are mainly dependent on FAO such as the anti-inflammatory Tregs (Berod et al., 2014; Newton et al., 2016; Cluxton et al., 2019). This hypothesis is supported by our data showing that GW0742 increased the polarization of $CD4^+$ Tregs *in vitro* (unpublished) and the prevalence of Tregs in lymph nodes (LNs) during weight loss in trained mice (Le Garf et al., 2019). Lifelong aerobic training has been regarded as a preventive strategy by improving the anti-inflammatory environment and by allowing the maintenance of circulating Tregs with aging (Minuzzi et al., 2019). However, whether these effects are driven by modifications of T cell metabolism is unknown. If improving PPARβ/δ activity could increase a Tregs phenotype, probably by increasing $CD4^+$ T cell FAO, it could also induce a metabolic inflexibility that would impair the migration capacity of Tregs in inflamed tissues which has been shown to depend on glycolysis (Kishore et al., 2017). This Tregs migration impairment could be deleterious notably in the context of SKM regeneration and SKM mass maintenance. SKM regeneration is driven during the early stage after injury by an increasing and coordinate infiltration and activity of immune cells (Tidball, 2017). The accumulation of Tregs shown at day 4 post-injury (Burzyn et al., 2013; Schiaffino et al., 2016), dampening inflammation (Panduro et al., 2018), profoundly declined with age paralleling a degradation of repair and regeneration processes (Kuswanto et al., 2016). This could be underpinned by the reduced mobilization of Tregs from the LNs of aged mice and/or their diminished recruitment to injured muscle (Kuswanto et al., 2016). Indeed, Tregs were shown to accumulate in secondary lymphoid organs with aging (Darrigues et al., 2018; Durand et al., 2018).

In this study, we asked the basic question of whether the invalidation of PPARβ/δ specifically in T cells alters the T cell profile in lymphoid organs and peripheral tissues. To do so, we investigated whether the age-related alteration of Tregs population was modified with this specific invalidation and whether this could alter the maintenance of SKM mass and physical capacities.

MATERIALS AND METHODS

Mice

Lck-Cre mice (Cre recombinase under control of the T cell-specific Lck gene promoter) were obtained from Jackson Laboratory [B6.Cg-Tg(Lck-cre)548Jxm/J, stock number 003802]. B6.Ppard^{TM1Mtz} mice (that possess loxP sites up- and downstream of PPARβ/δ exon 4) were previously generated (Schuler et al., 2006). Both strains are on the C57BL/6J background and were crossed to obtain T cell-specific PPARβ/δ knockout mice (named KO-T PPARβ/δ mice). It has previously been reported that Cre expression in Lck-Cre mice results in off-target effects including a decrease in thymic cellularity (toxic to $CD4^+CD8^+$ cells) (Shi and Petrie, 2012). Therefore, hemizygous Lck-Cre mice were used as controls. We used young (12–16 weeks) and older mice (39–45 weeks) of both sexes. Animals were maintained in a 12-h light, 12-h dark cycle and received food [A04 from UAR (Usine d'Alimentation Rationnelle), Villemoisson sur Orge, France] and water *ad libitum* (agreement number of the animal facility: A 06-088-014). All experimental procedures were conducted according to French legislation and to the EU Directive 2010/63 for animal experiments and were approved by the Institutional Ethic Committee for the Use of Laboratory Animals (CIEPAL-AZUR; N°2018110914193037).

Acute Muscle Injury

Mouse SKM were injured by injection of 50 μl of cardiotoxin (CTX) from *Naja pallida* at 0.03 mg/ml (L8102, Latoxan, France) in the left *Tibialis Anterior* (TLA), under gas anesthesia (5% Vetflurane). The right TLA (control leg) received 50 μl of saline solution (0.9% NaCl). In order to avoid excessive pain, mice received a subcutaneous injection of buprenorphine (100 μl at 30 μg/ml) 20 min before anesthesia. Before the killing, occurring at days 2 and 4 post-injury by intracardiac puncture, mice received an intraperitoneal injection of ketamine/xylazine (100 and 16 mg/kg, respectively). Different tissues (TLA and other SKM, heart, liver, spleen, lymph nodes, thymus, brown, and white adipose tissues) were harvested, weighed (except lymph nodes), and used for further analyses (see below).

Physical Test and Body Composition Measurements

Physical tests and body composition measurements were performed 2 days before CTX injection.

Mice Endurance Evaluation

The endurance of the mice was evaluated using a treadmill running test (five-lane motorized treadmill, LE8710 M, Bioseb) with a slope of 5°. During a warm-up phase, the speed of the treadmill was progressively increased every 2 min for 10 min (5–25 cm/s). This phase was followed by an acute exercise phase where the speed of the treadmill was increased by 5 cm/s every 15 min (30–40 cm/s) until the mice showed signs of exhaustion. The rear of the treadmill was equipped with a low-voltage electric stimulating bar to encourage each mouse to run. The bar was set to deliver 0.2 mA at a frequency of 0.25 Hz, which caused an uncomfortable shock but did not injure the animal. Number of shocks was recorded, and the electric delivery was stopped if 50 shocks were reached. The mice were previously familiarized with the tests 1 week before the evaluation.

Skeletal Muscle Strength Evaluation

The strength of upper limbs was measured using a grip test equipped with a bar (Bio-GS3, Bioseb). After three measurements, the best value was recorded and the maximal strength was expressed in Newton per gram (N/g).

Body Composition

Lean and fat masses were measured using an NMR Benchtop System (Minispec, Bruker France SAS). This instrument, which uses low-frequency (7.5 MHz) nuclear magnetic resonance, provides non-invasive examination of living animals such as mice with reduced animal stress, allowing measurements of fat tissue, lean tissue, and free fluid composition.

Skeletal Muscle Dissociation

Of each TLA (injured and control), 3/4 were washed with phosphate-buffered saline (PBS), minced, and digested with 2 mg/ml of type A collagenase in 1.5 ml of DMEM medium supplemented with 10% fetal bovine serum (FBS) for 60 min at 37°C. The muscle was further dissociated by performing five passages through a 3-ml syringe with an 18 G needle. After an additional 15 min of digestion and a second round in the syringe, the homogenate was diluted three times in DMEM (10% FBS), filtered (70 μm filter), and centrifuged at 300 × *g* for 20 min. The cell pellet was resuspended in PBS supplemented with 0.5% FBS at a final concentration of 5×10^6 cells/ml.

Fatty Acid Oxidation Assay of $CD4^+$ T Cells

Spleen and lymph nodes of control and KO-T PPARβ/δ mice were processed (either using a gentle MACS Dissociator and appropriate gentle MACS C tubes provided by the manufacturer (Miltenyi Biotec) for spleen, or pestle and mortar for lymph nodes), in order to obtain cell suspensions in PBS, pH 7.2, containing 0.5% FBS and 2 mM EDTA. Cell suspensions from both organs were pooled before continuing with the isolation of $CD4^+$ cells by magnetic labeling and separation using CD4 (L3T4) microbeads and LS or MS columns, respectively, following the manufacturer protocols (Miltenyi Biotec). The isolated $CD4^+$ cells were cultured at a concentration of 4×10^5 cells/well in a 48-well plate in RPMI containing 10% FCS, 100 units/ml penicillin/streptomycin, and 50 μM 2-mercaptoethanol. Cells were activated with anti-CD3/anti-CD28 beads (Dynabeads mouse T-activator CD3/CD28, Invitrogen) following instructions provided by the manufacturer. For certain conditions, 50 μM etomoxir (Sigma) was added as well. After 48 h, media were refreshed and 10 μl/well of a mix of radioactive and non-radioactive palmitate coupled to BSA (2:1 ratio; 15 μM fatty acid-free BSA (Sigma), 30 μM Na-palmitate (Sigma), and 10 μCi [0.83 μM [^{9,10}]-3H-palmitic acid (Perkin Elmer)] was added to each well. The radioactive and non-radioactive palmitate was coupled to BSA by first quickly adding the non-radioactive palmitate preheated at 70°C to BSA preheated at 50°C, followed by addition of the radioactive palmitate at this mix at 50°C. After a 24-h additional incubation, 100% trichloroacetic acid (10% final) was added to the cell suspensions and proteins were allowed to precipitate. After centrifugation, NaOH (final concentration 0.75 M) was added to the supernatant to increase pH to 12. Subsequently, 400 μl of supernatant was applied to ion-exchange columns (Dowex 1 × 8–200, Sigma), and ^3H_2O was recovered by eluting with 2.5 ml of H_2O. A 0.75-ml aliquot was then used for scintillation counting. Results were expressed as counts per minute (CPM) per 10^6 cells.

Cell Preparation and Flow Cytometry Analysis

All flow cytometry staining steps were performed at 4°C in the dark. Cell suspensions obtained as described above were incubated with fluorescently labeled primary antibodies for 20 min in PBS, 0.5% FBS, and 2 mM EDTA (FACS buffer). The following antibodies from eBioscience were used to analyze T cell populations in the thymus: CD3-fluorescein isothiocyanate, CD3-phycoerythrin, CD4-allophycocyanin, TCRβ-phycoerythrin-Cy7, TCRγδ-phycoerythrin, CD44-phycoerythrin-Cy7, CD62L-fluorescein isothiocyanate, CD25-phycoerythrin, and CD44-phycoerythrin. CD8-Peridinin chlorophyll antibody and Fc Block (antimouse CD16/CD32 monoclonal antibody) were from BD Biosciences. The following antibodies from Miltenyi were used to analyze T cell populations in lymph nodes and in SKM: CD3-fluorescein isothiocyanate, CD4-allophycocyanin-vio770, CD25-phycoerythrin, and FoxP3-allophycocyanin. After cell surface staining, cells were washed with FACS buffer. For intracellular staining of FoxP3, "FoxP3 staining buffer set" from Miltenyi was used according to manufacturer's protocol. Briefly, cells were incubated in 1× fixation/permeabilization solution for 30 min. Cells were then washed with FACS buffer, centrifuged, and resuspended in 1× perm solution containing FoxP3-APC antibody. After 30 min, cells were washed with 1× perm solution, centrifuged, and resuspended in FACS buffer. Flow cytometry acquisition was performed using a BD FACSCanto II flow cytometer (BD Biosciences), and data analyzed using FlowJo software.

RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted from cells or tissues with Trizol reagent (Invitrogen) and 1 μg of RNA was reverse-transcribed using a QuantiTect Reverse Transcription Kit (Qiagen) on a QcyclerII. Quantitative PCR was done using SYBR Premix Ex Taq (Tli RNase H Plus) (Ozyme) on a StepOne machine (Life Technologies). The mRNA levels of all genes were normalized to 36B4 transcript levels. Sequences of primers used are as follows: *Ppar*β/δ-F: GCA-GCC-TCA-ACA-TGG-AAT-GTC; *Ppar*β/δ-R CAT-ACT-CGA-GCT-TCA-TGC-GG. These primers allow the detection of intact exon 4 (wild-type form) and does not detect the recombined transcript, deleted from this exon (mutated form). *Cpt1a*-F: CTC-AGT-GGG-AGC-GAC-TCT-TCA; *Cpt1a*-R: GGC-CTC-TGT-GGT-ACA-CGA-CAA; *36B4*-F: TCC-AGG-CTT-TGG-GCA-TCA; and *36B4*-R: CTT-TAT-CAG-CTG-CAC-ATC-ACT-CAG-A.

Statistical Analyses

The results are presented as means ± standard deviations. All data were analyzed using Statview and GraphPad Prism v 5.0 software (San Diego, CA, United States). For each dependent variable under consideration, and according to assumptions for statistical analysis (i.e., normal distribution, equal variance), we performed: (1) non-parametric Mann–Whitney *U*-test to investigate the effect of genotype in young animals; (2) two-way ANOVA analyses to investigate independent effects of genotype and aging and the interaction effects between genotype and aging; (3) two-way ANOVA analyses to investigate the interaction effect between SKM injury (according to time of killing) and genotype. For this last analysis, the contralateral uninjured leg was used as control. We verified that no difference was shown according to gender of mice. This was not the case except for thymus, spleen, and VAT mass. For analysis of this variable, gender was taken into account as an additional factor in ANOVA analysis to isolate independent effect of genotype, aging and interaction effect between genotype and aging. Fisher PLSD and Newman-Keuls *post hoc* tests analyses were performed for multiple comparisons when statistical significance was reached for interaction effects. Statistical significance was accepted at $p < 0.05$.

RESULTS

Specific T Cell PPARβ/δ Invalidation Had No Effect on Thymic T Cell Development but Increased CD4$^+$ T Cell Prevalence in Lymph Nodes

We used a mouse model invalidated for PPARβ/δ specifically in T cells (KO-T PPARβ/δ) and verified that non-recombined *Ppard* mRNA levels (still containing exon 4) were lower in CD3$^+$ T cells from spleen and LNs of young KO-T PPARβ/δ mice compared with young control mice (**Figure 1A**). We would like to point out that the remaining non-recombined *Ppard* transcript levels measured in the knockout cells were only detected after an average of 34 PCR cycles, if at all, suggesting that remaining PPARβ/δ expression was non-existent to very low (Nadra et al., 2006). Thymus weight (**Table 1**) and cell count (data not shown) were not different in KO-T PPARβ/δ mice compared with the control mice. Thymic T cell development was not altered in KO-T PPARβ/δ mice. Indeed, cytometric analyses of isolated thymic T cells showed that young KO-T PPARβ/δ mice exhibit similar CD3$^+$ T cells percentage and double negative (DN) subsets prevalence, compared with the control animals (**Figure 1B**). Flow cytometry analysis performed on isolated T cells from LNs showed that the percentage of CD4$^+$ T cells in LNs was significantly higher in young KO-T PPARβ/δ mice compared with the control animals (**Figure 1C**). mRNAs of *Ppard*β/δ and *Cpt1a*, the main PPARβ/δ target-gene involved in FAO in lymphoid tissues (Mothe-Satney et al., 2016) were decreased in LNs of KO-T PPARβ/δ mice (**Figure 1D**). In addition, treatment of purified T cells from KO-T PPARβ/δ mice with a specific PPARβ/δ agonist (GW0742) was not able to induce *Cpt1a* expression, confirming loss of PPARβ/δ function. This result is part of an upcoming manuscript regarding a role for PPARβ/δ in Treg polarization. Moreover, palmitate oxidation by lymphoid organ-isolated CD4$^+$ T cells from KO-T PPARβ/δ mice tended to be lower than those from control mice. Interestingly, this level of oxidation was comparable with the one observed in CD4$^+$ T cells from control animals treated with etomoxir, an inhibitor of Cpt1a (O'Connor and Milone, 2020; **Figure 1E**).

Increased Accumulation of CD4$^+$ T Cells in Skeletal Muscle in the Early Stage of Regeneration in KO-T PPARβ/δ Mice

We induced a myonecrosis with CTX injection in TLA to cause an immune challenge which is known to be accompanied by an accumulation of CD4$^+$ T cells in injured SKM (Burzyn et al., 2013). At days 2 and 4 after the acute injury (i.e., the early stage of the inflammatory process), we collected LNs and digested TLA [injured and uninjured (control)]. As naïve T lymphocytes circulate through the lymph-vascular system and enter and exit lymphoid organs, at this early stage of the inflammatory process, we investigated whether both the proportion of LN-resident CD4$^+$ T cells and the number of CD4$^+$ T cells in SKM were altered in young KO-T PPARβ/δ mice. Regarding the prevalence of CD4$^+$ T cells in LNs, the two-way analysis of variance ANOVA test (injury, genotype) showed a significant interaction effect ($p < 0.0001$) between the injury time and the genotype of mice (**Figure 2A**). As described before, the percentage of LN CD4$^+$ T cells at baseline was significantly higher in KO-T PPARβ/δ compared with the control animals. However, this percentage was significantly lower at day 2 post-injury while it returned to baseline at day 4 in KO-T PPARβ/δ mice. In control mice, this percentage of CD4$^+$ T cells did not significantly vary with injury. This showed a transient mobilization of CD4$^+$ T cells in response to this challenge only in KO-T PPARβ/δ mice (**Figure 2A**). In order to investigate the concomitant difference of the presence of CD4$^+$ T cells in the injured TLA at day 2 according to the genotype of mice, we retrieved the stromal vascular fraction which was then characterized by flow cytometry. At day 2, CD4$^+$ T cell number (per mg of TLA) was significantly

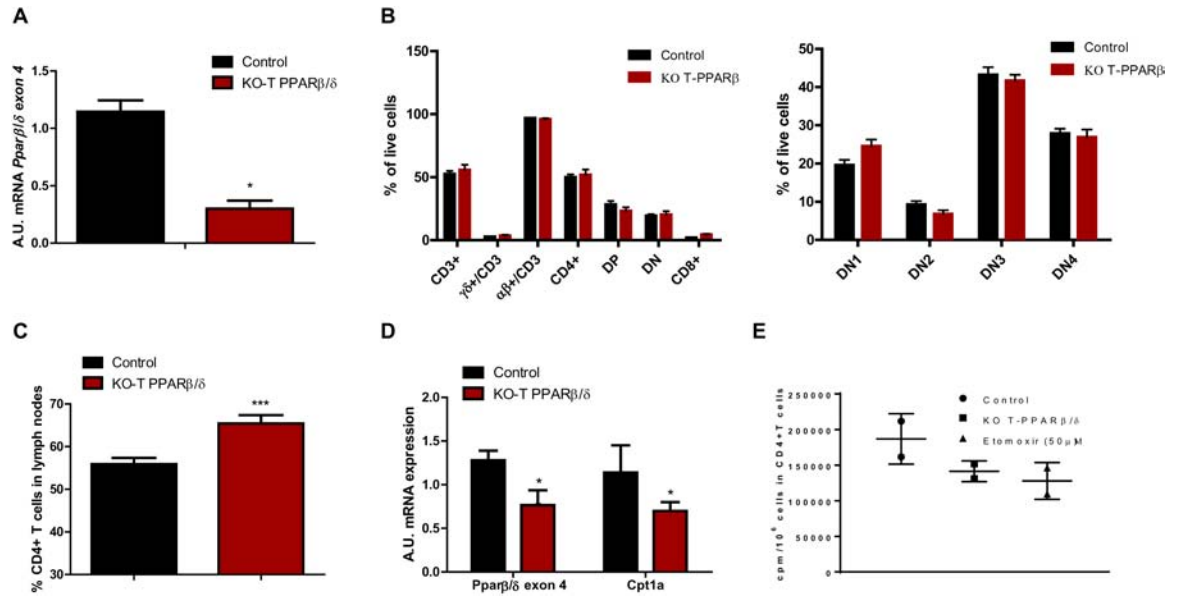

FIGURE 1 | Characterization of T cells in thymus and lymph nodes of young KO-T PPARβ/δ mice compared to young control mice. **(A)** *Pparβ/δ exon 4 mRNA level in T cells*. Relative *Pparβ/δ* mRNA levels in isolated $CD3^+$ T cells from spleen and lymph nodes. Isolation of $CD3^+$ cells was performed using magnetic labeling according to the manufacturer protocol. RNA was extracted as described in "Materials and Methods"; *36B4* has been used as housekeeping mRNA; $n = 3$ in each group. **(B)** *T cell population analysis in thymus and thymic development of T cells*. Cell suspensions from thymus were obtained as described in "Materials and Methods" for cell suspensions from spleen. Left panel, percentages of the different T cell populations in thymus obtained from flow cytometry analyses are shown: $CD3^+$ (and among $CD3^+$, relative percentages of TCR- αβ and TCR- γδ positive T cells), $CD4^+$, $CD8^+$, DN ($CD4^-CD8^-$), DP ($CD4^+CD8^+$). Right panel, relative percentages of DN1 ($CD25^-CD44^+$), DN2 ($CD25^+$ $CD44^+$), DN3 ($CD25^+$ $CD44^-$), and DN4 ($CD25^-CD44^-$) among DN cells are indicated ($n = 6$ par group). **(C)** *$CD4^+$ T cell prevalence in lymph nodes in $CD3^+$ T cells*. Cell suspensions from lymph nodes were obtained as described in "Materials and Methods." Flow cytometry analyses were performed to analyze $CD4^+$ in $CD3^+$ cell gating (control, $n = 11$; KO-T PPARβ/δ, $n = 8$). **(D)** *Pparβ/δ exon 4 and Cpt1a mRNA expression in lymph nodes*. RNA was extracted from lymph nodes cell suspension as described in "Materials and Methods." The relative amounts of *Pparβ/δ and Cpt1a* mRNA were quantified using *36b4* as housekeeping mRNA (control, $n = 3$; KO-T PPARβ/δ, $n = 5$). **(E)** *Palmitate oxidation of isolated $CD4^+$ T cells*. FAO was measured as ^{3}H-palmitate conversion to ^3H_2O and quantified as CPM/10^6 cells in *in vitro*-activated $CD4^+$ cells treated or not with 50 μM etomoxir ($n = 2$ per group). Values are presented as box and whiskers min to max. Except for **(E)**, values are mean ± SD. Statistical analyses were performed using Mann–Whitney *U*-test.*$p < 0.05$, different from control mice.

TABLE 1 | Effects of age and genotype on body composition.

	Control Cre ($n = 28$)		KO T PPARβ /δ ($n = 27$)		Genotype*age
	Young mice	Old mice	Young mice	Old mice	
Body weight (g)	22.7 ± 4.4	30.1 ± 5.8*	22.4 ± 2.6	27.3 ± 3.9*	n.s.
Lean mass (g)	15.3 ± 2.8	16.6 ± 1.7*	15.0 ± 1.9	18.4 ± 3.2*	n.s.
Fat mass (g)	4.4 ± 1.3	8.1 ± 3.9*	4.7 ± 1.0	5.5 ± 1.4*	$F = 8.3$; $p = 0.006$
Thymus (mg/g)	1.9 ± 0.6	1.1 ± 0.2*	1.7 ± 0.7	1.0 ± 3.0*	n.s.
Spleen (mg/g)	3.9 ± 0.8	3.5 ± 0.9	3.0 ± 0.9	3.8 ± 1.0	$F = 9.2$; $p = 0.004$
Heart (mg/g)	5.2 ± 0.6	4.6 ± 0.5	5.5 ± 0.5§	5.8 ± 0.8§	$F = 6.8$; $p = 0.010$
TLA (mg/g)	1.7 ± 0.17	1.42 ± 0.2*	1.93 ± 0.3§	1.58 ± 0.3*§	n.s.
Liver (mg/g)	43 ± 19	44 ± 7	49 ± 4	45 ± 6	n.s.
VAT (mg/g)	11.2 ± 0.6	24.9 ± 1.28*	13.3 ± 0.4§	13.2 ± 0.5§	$F = 16.6$; $p = 0.0002$
BAT (mg/g)	3.8 ± 0.2	4.8 ± 0.4*	3.9 ± 0.11	4.8 ± 0.2*	n.s.

*Statistical analysis was performed using a two-way (age and genotype) analysis of variance (ANOVA). *$p < 0.05$, age effect; § $p < 0.05$, genotype effect. Data are expressed as the mean ± SD in each group. Young control (n = 21); young KO-T PPARβ/δ (n = 15); older control (n = 7); older KO-T PPARβ/δ (n = 12). TLA, tibialis longitudinus anterior skeletal muscle; BAT, brown adipose tissue; VAT, visceral adipose tissue.*

higher in injured TLA compared with uninjured TLA. Flow cytometry analysis showed that relatively few T lymphocytes were detected in uninjured TLA. The difference of their number was not significant between KO-T PPARβ/δ and control mice (**Figure 2B**). In contrast, in injured TLA muscle, $CD4^+$ T cell number increased with a significantly higher magnitude in KO-T PPARβ/δ mice compared with the control mice (**Figure 2B**). The $CD3^+CD8^+$ T cells increase was not different between

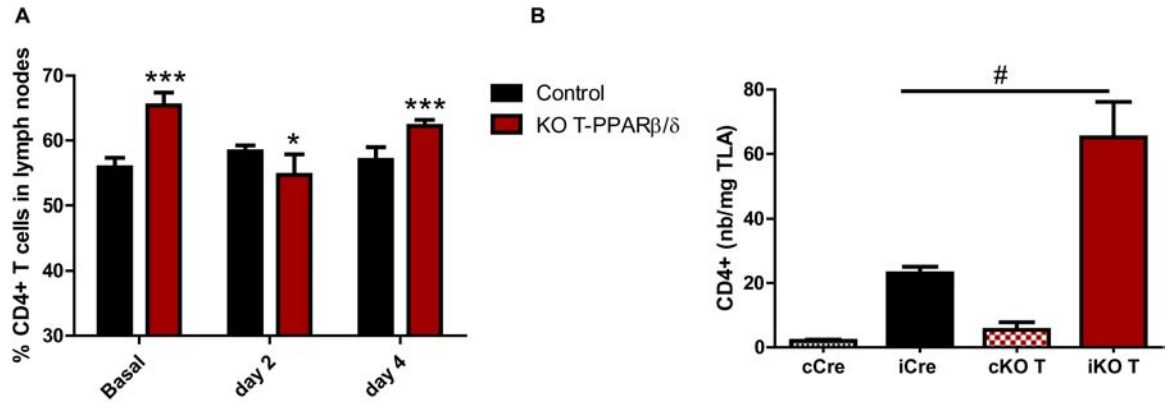

FIGURE 2 | $CD4^+$ T cell prevalence in lymph nodes and in TLA after cardiotoxin-induced injury of young KO-T PPARβ/δ and control mice. **(A)** *$CD4^+$ T cell prevalence in lymph nodes*. Percentages of $CD4^+$ T cells among $CD3^+$ cell population in basal state and at day 2 (n = 5 per group) and day 4 (control, n = 8; KO-T PPARβ/δ, n = 6) after injury of TLA with cardiotoxin. **(B)** *$CD4^+$ T cell number in TLA*. Number of $CD4^+$ T cells among $CD3^+$ T cell population (expressed per mg of TLA) in cells from digested TLA 2 days after injury with cardiotoxin. Left TLA of each mouse was injured and the right TLA of the same mice was injected with saline (cCre: uninjured TLA (right) of control Cre mice; iCre: injured TLA (left) of control Cre mice; cKO T: uninjured TLA (right) of KO-T PPARβ/δ mice; iKO T: injured TLA (left) of KO-T PPARβ/δ mice). Values are mean ± SD. Statistical analysis was performed using two-way (time post-injury and genotype) analysis of variance (ANOVA) test. Interaction effect between time post-injury and genotype was significant.*$p < 0.05$, ***$p < 0.0005$ different from control mice at the same time (basal, day 2, day 4); $^{\#}p < 0.05$ interaction effect between genotype and injury.

groups (data not shown). These results show that in young mice, invalidation of PPARβ/δ in T cells modifies both the prevalence of $CD4^+$ in LNs and their accumulation in injured SKM.

Invalidation of PPARβ/δ in T Cells Decreased the Age-Related Sequestration of Tregs in Lymph Nodes

Among $CD4^+$ T cells, the population of Tregs is the highest mobilized in challenged SKM (Burzyn et al., 2013) but to a smaller extent with aging (Kuswanto et al., 2016). As aging has been repeatedly shown to increase $CD4^+$ Treg population sequestration in LNs (Nikolich-Zugich, 2014) and knowing that a majority of $CD4^+$ Tregs in LNs are resident and not circulating T cells in old mice (Durand et al., 2018), we next investigated whether invalidation of PPARβ/δ in T cells modified the prevalence of Tregs in LNs of both young and older animals. The transcription factor FoxP3 is essential for specifying the lineage and immune suppressive function of Tregs (Fontenot et al., 2003). No effect of the genotype was evidenced in young mice on the prevalence (**Figure 3A**) or mean intensity fluorescence (MFI) (not shown) of $FoxP3^+$ T cells in LNs. Older control mice had a significantly higher percentage of $FoxP3^+CD4^+$-labeled T cells in LNs (**Figure 3A**), but no difference was observed for MFI (not shown). However, as evidenced by the two-way ANOVA test, a significant interaction effect between the two factors "aging" and "genotype" was shown. In older KO-T PPARβ/δ mice, the prevalence of $FoxP3^+CD4^+$ T cell was not different from those characterized in young mice (**Figure 3A**). Tregs constitutively express CD25, the α subunit of the IL-2 receptor. IL-2 signaling is critical for maintaining the homeostasis and competitive fitness of Tregs (Fontenot et al., 2003). The prevalence of $CD25^-$ T cells among $CD4^+FoxP3^+$ T cells has been shown to be higher in aged animals compared with young animals, and this population has been shown to become functionally heterogeneous with aging (Nishioka et al., 2006). CD25 staining of $FoxP3^+$ T cells was performed to differentiate $FoxP3^+CD25^-$ and $FoxP3^+CD25^+$ subpopulations. An independent increase with age was shown by two-way ANOVA analysis for $FoxP3^+CD25^-$ cell prevalence. Moreover, a significant independent genotype effect was shown for both prevalence of $FoxP3^+CD25^-$ and $FoxP3^+CD25^+$ which were, respectively, lower and higher in KO-T PPARβ/δ animals (**Figure 3B**). $FoxP3^+CD25^-$ population was significantly lower in older KO-T PPARβ/δ compared with age-matched control mice (**Figures 3B,C**).

Together, our results show a preventive effect of the invalidation of PPARβ/δ in T cells against age-induced increase in Tregs prevalence in LNs, with a preservation of the population of $FoxP3^+CD25^+$ cells.

The Invalidation of PPARβ/δ in T Cells Prevented the Age-Related Change of Body Composition and the Loss of Endurance Capacity

Aging is characterized by a progressive deterioration of physiological systems, and the loss of muscle mass is one of the most recognizable, leading to muscle weakness and mobility impairments. The link between immune change with aging and muscle mass maintenance is not clear. We basically asked ourselves the question of whether the invalidation of PPARβ/δ specifically in T cells would have repercussion on body composition and exercise capacities in both young and older mice. We performed NMR analysis of body composition. Lean mass was significantly lower (**Figure 4A** and **Table 1**), and body mass and fat mass were significantly higher in older control mice compared with young animals (**Figure 4B** and **Table 1**). Interaction effects between genotype

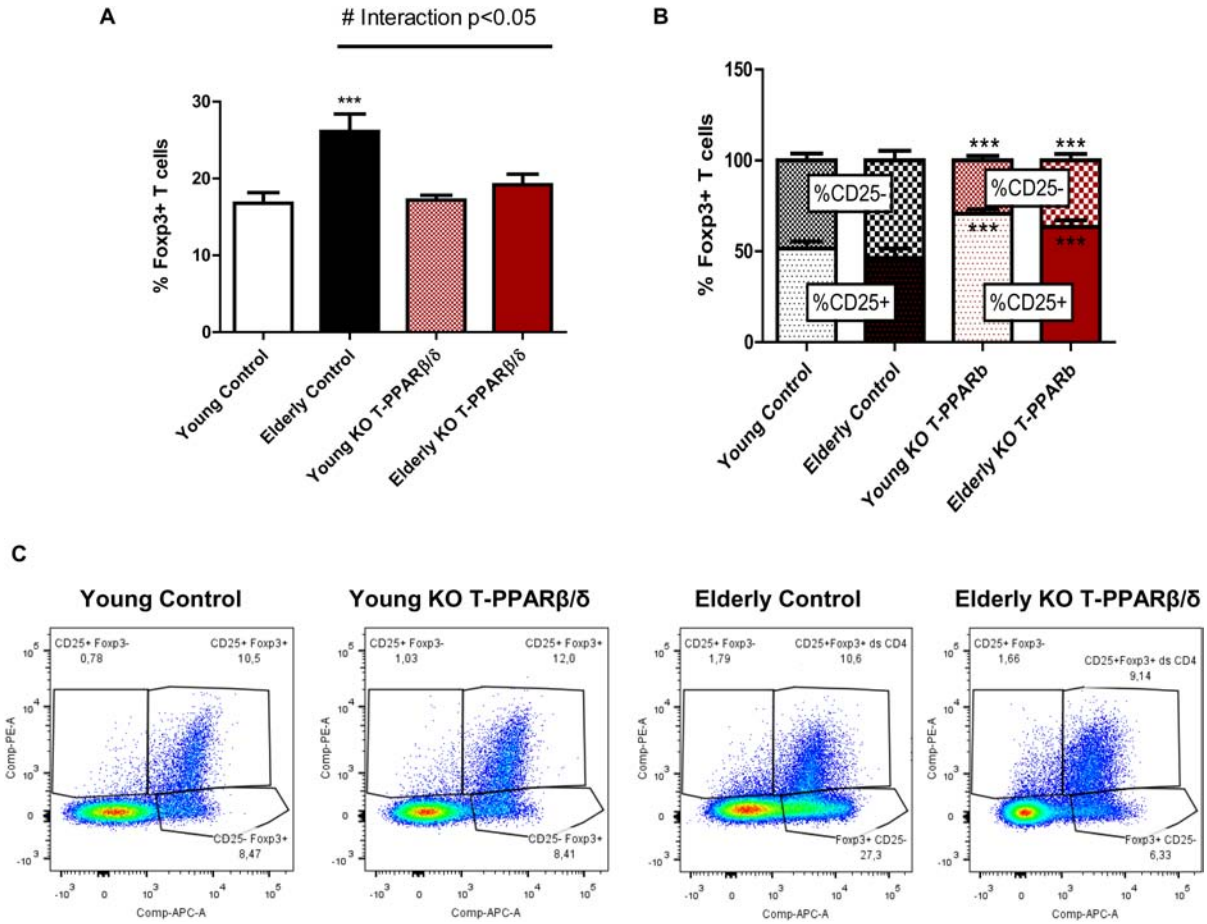

FIGURE 3 | Invalidation of PPARβ/δ in T cells preserves prevalence of FoxP3$^+$ Tregs in lymph nodes with age. Percentage of **(A)** labeled FoxP3$^+$ Tregs in CD3$^+$CD4$^+$ T and **(B)** of the subpopulations CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ of FoxP3$^+$ Tregs cells, in young and older mice invalidated for PPARβ/δ in T cells (KO-T PPARβ/δ) or not (control). **(C)** Representative flow cytometry dot plots of CD4$^+$CD25$^+$FOXP3$^+$ cells and CD4$^+$CD25$^-$FOXP3$^+$ cells among the CD3$^+$CD4$^+$ T cells in lymph nodes of all mice. Values are mean ± SD. Statistical analysis was performed using a two-way (age and genotype) analysis of variance (ANOVA). $^{\#}p < 0.05$, interaction effect between age and genotype; $^{***}p < 0.005$, different from age-matched control mice. Young control (n = 11); young KO-T PPARβ/δ (n = 8); older control (n = 7); Older KO-T PPARβ/δ (n = 12).

and aging were significant for both lean and fat mass but not for body weight. Indeed, older KO-T PPARβ/δ mice had significant higher lean mass (**Figure 4A**) and lower fat mass (**Figure 4B**) compared with their older control counterparts. Strikingly, lean and adipose tissue masses of older KO-T PPARβ/δ mice were not different from those of young animals (**Figure 4B**).

Next, tissues that were taken during the killing were weighted (**Table 1**), and their weight normalized to the body weight of animals to correct for intermice variability. Moreover, we considered gender a covariable to avoid a confounding effect when necessary. An effect of age was observed on thymus and BAT weights which were decreased for both genotypes (**Table 1**). However, older KO-T PPARβ/δ mice did not exhibit the decreasing effect of age on the mass of spleen and heart and the increasing effect of age on VAT mass (**Table 1**). No difference between genotypes was evidenced for liver, thymus, spleen, and for BAT, but TLA SKM and heart masses were both significantly higher in KO-T PPARβ/δ mice compared with control mice (**Table 1**). These results suggest that the effect of genotype on lean mass is mainly due to a muscle mass maintenance.

To capture a physical capacity profile of these mice, we undertook assessments for strength and endurance analysis. Our data did not reveal marked differences according to the genotype of mice in maximal voluntary grip strength which was, however, significantly lower in older mice compared with young animals (**Figure 4C**). In contrast, treadmill endurance performance was significantly higher in KO-T PPARβ/δ mice (**Figure 4D**). The difference was two times higher than their "control" older counterparts despite that it might be underestimated, as we have stopped the test after 1 h of running as requested by the institutional ethic committee for the use of laboratory animals. This last result suggests that KO-T PPARβ/δ mice are protected against an age-related decrease in running capacity despite having never being trained.

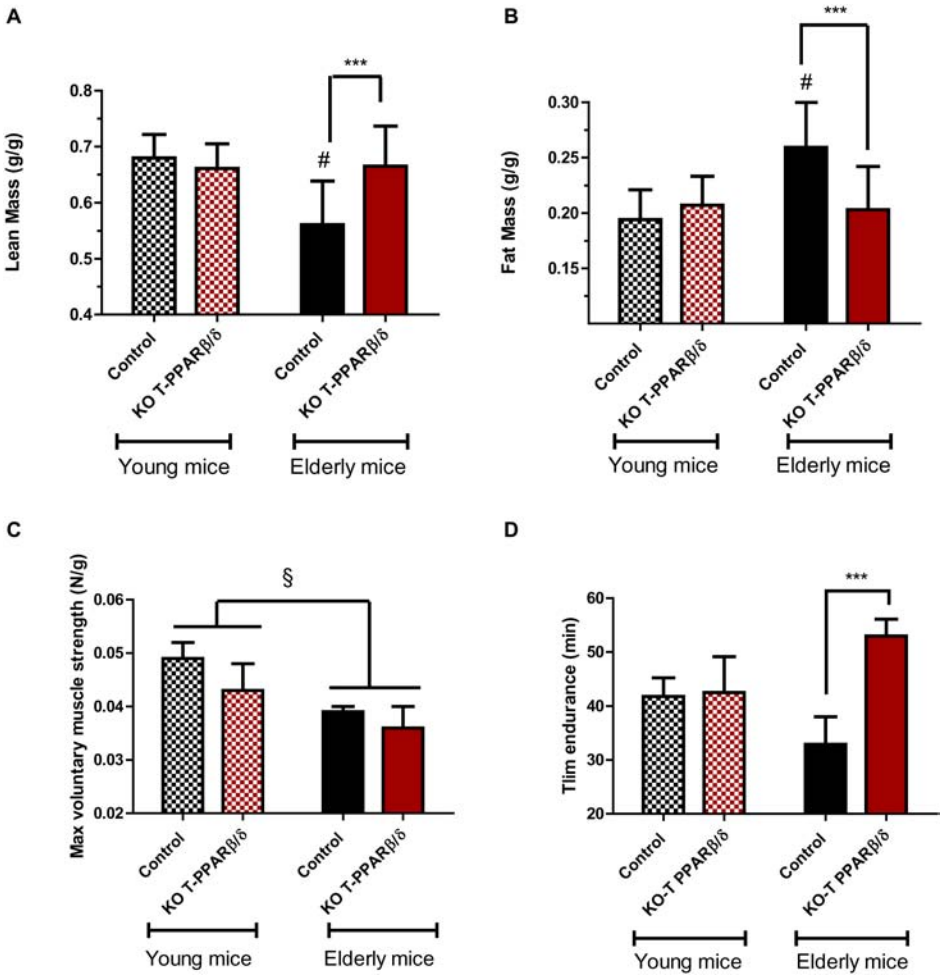

FIGURE 4 | Invalidation of PPARβ/δ in T cells preserves body composition and endurance capacities with age. Comparison of **(A)** lean and **(B)** fat mass, per weight of mice, according to age (young: 12–16 weeks, striped bars; older; 39–45 weeks, full bars) and genotype. Effect of age and genotype on **(C)** baseline maximal voluntary grip strength (*N* per g of body weight) and on **(D)** exhaustive treadmill running time (Tlim). Values are mean ± SD. Statistical analysis was performed using a two-way (age and genotype) analysis of variance (ANOVA). $^{\#}p < 0.05$, different from control young mice; $^{***}p < 0.005$, different from age-matched control mice. § $p < 0.05$, different from young animals. Young control ($n = 11$); young KO-T PPARβ/δ ($n = 8$); older control ($n = 7$); older KO-T PPARβ/δ ($n = 12$).

DISCUSSION

PPARβ/δ is a key player in the transcriptional regulation of lipid metabolism but less well studied than other subtypes (i.e., PPARα and PPARγ) in T cells. Contrary to an activation of the pathway by its specific agonist GW0742 (Mothe-Satney et al., 2016), we showed in this study that the invalidation of PPARβ/δ specifically in T cells decreased FAO potential of $CD4^+$ T cells. Moreover, KO-T PPARβ/δ mice had no significant defect in thymus cellularity and T cell development. By using a mouse model of global invalidation of PPARβ/δ, Zhao et al. (2018) showed defects in PPARβ/δ-deficient thymocytes. This apparent contradiction suggests that PPARβ/δ expressed in other cell types than T cells in the thymic environment would be mainly responsible for this phenotype. In contrast, we have previously shown that the overexpression of PPARβ/δ specifically in T cells alters T cell development, suggesting that low or no expression of this nuclear receptor is more supportive of normal T cell thymic development and proliferation (Mothe-Satney et al., 2016). Most changes in T cells seemed to occur in the periphery in KO-T PPARβ/δ mice.

One of the main consequences of PPARβ/δ activation is to increase fatty acid metabolism. Interestingly, increased FAO of $CD4^+$ memory T cells—a subset of $CD4^+$ T cells that are able to use FAO for their energetic metabolism—could contribute to the decline in T cell functions with age and in their ability to survive (Yanes et al., 2019). In our study, *Cpt1a* was decreased in $CD4^+$ T cells and in LNs, due to the invalidation of PPARβ/δ. This decrease in *Cpt1a* seemed to induce a concomitant decrease in FAO in isolated $CD4^+$ T cells and was associated with a higher prevalence of $CD4^+$ T cells in LNs of young KO-T PPARβ/δ mice. Based on genetic models of Cpt1a deletion in T cells, Raud et al. (2018) have demonstrated that in the absence of Cpt1a, T effector cells, memory T cells, and Treg cell

development and function occur normally. This indicates that Cpt1a is dispensable and that these cells could adapt metabolically to a reduction in long chain (LC)-FAO (Raud et al., 2018). This could also explain why the prevalence of Tregs was unchanged in LNs of young animals. However, with aging, Tregs were shown to accumulate in secondary lymphoid organs (Darrigues et al., 2018; Durand et al., 2018), but this effect of age was surprisingly not observed in KO-T PPARβ/δ mice. Effects of Cpt1a invalidation was not characterized in old animals in the study of Raud et al. (2018), but regarding our results and those from Yanes et al. (2019), it could be possible that LC-FAO would act as a determinant of the loss of T cell functions and survival with age. When further characterizing Tregs using FoxP3 and CD25 markers, we found a preserved functional $FoxP3^+CD25^+$ prevalence and a marked lower prevalence of $CD25^-$ cells in LNs from older KO-T PPARβ/δ mice. It has been shown that the absence of CD25 in peripheral Tregs was determinant of their increased susceptibility to oxidative stress, mitochondrial dysfunction, and apoptosis and of the transcription of lipid and cholesterol biosynthetic key enzymes (Toomer et al., 2019). Moreover, CD25 expression at the cell surface was dependent on a redox-regulated signaling pathway (Secchi et al., 2020) and was decreased on Tregs from aged mice (Chougnet et al., 2011). Taking all these data into account, since older KO-T PPARβ/δ mice display less $FoxP3^+CD25^-$ cells (accompanied by a concomitant increase in $FoxP3^+CD25^+$ cell proportion), one might expect that Tregs of these mice are preserved from the effects of aging and oxidative stress, and thus more functional and less prone to immunosenescence. The deletion of PPARβ/δ in T cells would potentially contribute to increase the stress-related pathway responses by maintaining NF-κB activity in aged mice, which could sustain naive T cell turnover essential to maintain naive T cell diversity including Tregs (Mold et al., 2019). Moreover, NF-κB p65 subunit, which physically interacts with PPARβ/δ (Schnegg et al., 2012), was demonstrated to be crucial for the expression of FoxP3 and for mature Tregs homeostasis in secondary lymphoid organs (Oh et al., 2017). While the exact mechanisms remain to be determined, our results suggest that Tregs of KO-T PPARβ/δ mice might be protected from the negative effects observed on Tregs fate with aging. This could have implications for the immunosuppression that occurs with aging.

PPARβ/δ drives redox and metabolic changes in T cells that could modify some of their properties such as their capacity to be mobilized in tissues. Recent data from the group of D. Mathis have shown that $CD4^+$ T cells present in injured mouse SKM are mainly Tregs, constituting 40 to 60% of the $CD4^+$ T cell compartment (Burzyn et al., 2013; Kuswanto et al., 2016). Interestingly, when we induced SKM injury to study local acute immune response, we observed an increase in the number of infiltrated $CD3^+CD4^+$ T cells in injured TLA of KO-T PPARβ/δ mice compared with control mice at day 2 post-injury. As the number of resident $CD4^+$ T cells remained unchanged in the uninjured TLA in both groups of mice, our data suggest an increase in their recruitment upon injury. This hypothesis is supported by a higher capacity of $CD4^+$ T cells of KO-T PPARβ/δ mice to be mobilized from lymph nodes, as suggested by the increase in $CD4^+$ T cells within SKM concomitant with a decrease of $CD4^+$ T cells in LN of KO-T PPARβ/δ mice. Observation of an increase in Tregs in injured SKM is generally made at day 4, when they reached a peak. The kinetic is quite similar to that of anti-inflammatory M2 macrophages (Burzyn et al., 2013; Kuswanto et al., 2016; Tidball, 2017). However, little is known about the type of $CD3^+$ T cells in injured SKM at earlier time points (day 2) corresponding to the peak of proinflammatory M1 macrophages. Immune cells are important sources of cytokines that can affect myogenesis and regulate SKM growth and regeneration (Fu et al., 2015; Tidball, 2017; Wang et al., 2018). We do not know if the infiltrated cells had improved the regeneration process itself but importantly, we show in this study that lean mass of KO-T PPARβ/δ mice was maintained with age. This lean mass maintenance was mainly related to a higher muscle mass (SKM and heart) compared with age-matched control animals. We recently obtained data (unpublished) showing that long-term treatment with GW0742 had a negative effect on SKM maintenance (mainly oxidative SKM). These results suggest that chronically activating PPARβ/δ pathway could be negative for the maintenance of SKM integrity, an effect which differed *a priori* from the short-term "exercise-mimetic" effect of its activation (Narkar et al., 2008).

It has been shown that many features of physiological aging (including sarcopenia) and the main features of immunosenescence, could be partly prevented by maintaining a high level of physical activity during life (Pollock et al., 2015; Duggal et al., 2018). Our older KO-T PPARβ/δ mice, similarly to young mice, maintained a surprising high running endurance. Normally, for a fixed running speed, oxygen consumption and duration of running decrease with aging in mice (Schefer and Talan, 1996). Furthermore, at exhaustion, the capacity to run of older KO-T PPARβ/δ mice was progressively declined, similarly to young animals, whereas older control mice refused to run as was previously reported (Schefer and Talan, 1996). Relationships between endurance performance and body weight or with the SKM oxidative adaptation marker citrate synthase, were not clearly evidenced in our study (**Supplementary Figure 1**), suggesting that exhaustion time in our mice is not principally related to these parameters. Exhaustion time is mainly a reflection of fatigue which is among the most common presenting complaints in older adults (Simonsick et al., 2016). Performance capacities and perceived fatigability—the two suggested attributes of fatigue—are modulated during exercise by multiple factors such as contractile capacities, motivation, core temperature, and pain (Enoka and Duchateau, 2016) which were not controlled in our study. It is clear that modification in T cells (i.e., PPARβ/δ deficiency) might play a role in a lifetime exercise-trained phenotype, but further research needs to be done to provide definite proof that T cell modulation affects endurance and to unravel the mechanisms by which it can do so.

Together, our data link modification of T cells to body composition and running performance. Even though we are aware of the various limitations of this study, we think that these surprising results lead to novel hypotheses for reducing age-related changes in muscle by manipulating T cells. Mechanisms

remain to be elucidated but many questions have emerged from this study. Did T cell secretome of KO-T PPARβ/δ mice lead to a rejuvenating secretome able to maintain immune system and body composition into old age? Did the sensitivity to stress or cytokines that are known to be involved in exercise adaptations increase? Did the expression of immune modulatory surface molecules change? Did the modulation of energetic substrates used create a nutritional environment favorable for the maintenance of physical capacity with age? Our study is the first to show that the modification of a transcriptional regulator of fatty acid metabolism in T cells could have a positive impact on the preservation of body composition and the capacity to run with aging despite an *a priori* sedentary behavior of mice. Among all the characteristics of mice advancing with age, we come to a rather striking set of observations leading us to believe that the invalidation of PPARβ/δ in T cells protects them from pathological aging, rendering these mice protected from susceptibility to age-related immune changes and frailty.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Comité Institutionnel d'Éthique Pour l'Animal de Laboratoire (CIEPAL)-AZUR n° 28; N° 2018110914193037.

AUTHOR CONTRIBUTIONS

A-SR and IM-S designed the research. A-SR and IM-S conducted research and analyzed data. WW provided B6.Ppard^{TM1Mtz} mice. JM, JN, BS, SL, and GL assisted with the experiments. A-SR and IM-S wrote the manuscript. JN, GC, BS, GL, and WW reviewed and edited the manuscript. All authors read and approved the final manuscript. The authors report no conflicts of interest.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.587753/full#supplementary-material

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