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Review

Macrophages in Skeletal Muscle Dystrophies, An Entangled Partner

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Abstract. While skeletal muscle remodeling happens throughout life, diseases that result in its dysfunction are accountable for many deaths. Indeed, skeletal muscle is exceptionally capable to respond to stimuli modifying its homeostasis, such as in atrophy, hypertrophy, regeneration and repair. In particular conditions such as genetic diseases (muscular dystrophies), skeletal muscle's capacity to remodel is strongly affected and undergoes continuous cycles of chronic damage. This induces scarring, fatty infiltration, as well as loss of contractibility and of the ability to generate force. In this context, inflammation, primarily mediated by macrophages, plays a central pathogenic role. Macrophages contribute as the primary regulators of inflammation during skeletal muscle regeneration, affecting tissue-resident cells such as myogenic cells and endothelial cells, but also fibro-adipogenic progenitors, which are the main source of the fibro fatty scar. During skeletal muscle regeneration their function is tightly orchestrated, while in dystrophies their fate is strongly disturbed, resulting in chronic inflammation. In this review, we will discuss the latest findings on the role of macrophages in skeletal muscle diseases, and how they are regulated.

Keyword: Macrophage, skeletal muscle, muscle dystrophy, repair, inflammation

ABBREVIATIONS

AMPK	AMP-activated Protein Kinase
BM	Bone Marrow
BMD	Becker Muscular Dystrophy
BMDM	Bone Marrow Derived Macrophages
C/EBP	CCAAT-enhancer-binding proteins
CCL	C-C chemokine ligand
CCR	C-C chemokine receptor
CK	Creatine Kinase
CRISPR/Cas9	Clustered Regularly Interspaced Short Palindromic Repeats
CSF	Colony Stimulating Factor
CTGF	Connective Tissue Growth Factor
CXCL	C-X-C chemokine ligand
CXCR	C-X-C chemokine receptor
DAMP	Damage-Associated Molecular Pattern
DGC	Dystrophin-Glycoprotein Complex
DTA	Diphtheria Toxin A
ECM	Extracellular Matrix
eMHC	embryonic myosin heavy chain
FAP	Fibro/Adipogenic Progenitor
GRMD	Golden Retriever Muscular Dystrophy
GR	Granulocyte Receptor
GM	Granulocyte Macrophage
IFNγ	Interferon Gamma
IGF	Insulin-like Growth Factor
IL	Interleukin
LGMD	Limb-girdle Muscular Dystrophy
LTBP	Latent TGFβ Binding Protein
LPS	lipopolysaccharide
MAA	Myositis-Associated Antibody
MAB	Mesoangioblast
MAPK	Mitogen-activated protein kinase

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MHC	Myosin Heavy Chain
MKP-1	Mitogen-activated protein kinase phosphatase 1
MMP	Matrix Metalloproteinase
MD	Muscular dystrophy
MSA	Myositis-specific antibody
NAMPT	Nicotinamide phosphoribosyl-transferase
Nfix	Nuclear Factor I X
NFκB	Nuclear Factor kappa B
NSAID	Nonsteroidal anti-inflammatory drugs
OPN	Osteopontin
PDGFR	Platelet Derived Growth Factor Receptor
RANKL	receptor activator of nuclear factor NF-κB ligand
RANTES	Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted
SC	Satellite Cell
SCG	sarcoglycan
TGFβ	Transforming growth factor beta
TIMD4	T Cell Immunoglobulin And Mucin Domain Containing 4
TNF	Tumor Necrosis Factor
Treg	regulatory T cell
uPA	urokinase plasminogen activator
VEGF	Vascular endothelial growth factor
WT	Wild Type

INTRODUCTION

Disruption of homeostasis in skeletal muscle tissue can be triggered by many variables, including changes in diet or exercise, local injury, systemic infection, genetic disease, and ageing. In most of these cases, inflammation and in particular macrophages play a primordial role in the pathology. Macrophages, along with neutrophils, are part of the leukocyte family first described in 1908 by Elie Metchnikoff. In addition to their immunoprotective role against pathogens, macrophages have a number of additional roles during development and tissue remodeling, in particular in skeletal muscle [1–3].

Skeletal muscle regeneration is made possible by a population of adult muscle stem cells called satellite cells (SC) [4]. After damage or microenvironment changes, SCs activate, proliferate, and differentiate into myoblasts/myocytes before fusing with one another or with surrounding myofibers. As fitting their definition as a stem cell, SCs are capable of self-renewal which maintains a potent pool of cells throughout life (reviewed in [5, 6]). Despite the essential role SCs play in muscle regeneration, these events won't happen without the help of other cells harbored in the interstices between the myofibers: endothelial cells, pericytes [7], fibro/adipogenic progenitors (FAP) [8–10], and foremost, macrophages [11–16]. Specifically, following damage, macrophages are responsible for the release of a pool of cytokines, chemokines and alarmins called damage-associated molecular pattern (DAMPs). These molecules are part of a coordinated response that initiates sterile inflammation and induction of blood-circulating monocytes' infiltration into the tissue.

In healthy tissue, successful skeletal muscle regeneration is typically complete within 3 weeks, and without any need for external intervention (anti-inflammatory drugs for example) [17]. However, when disrupted, the process can take months and is often associated with fibrotic deposition (scarring) and adipogenesis. Examples of such disruptions in muscle homeostasis include repeated injures, volumetric mass injuries, modification in homeostasis during ageing (sarcopenia), prolonged immobility/atrophy (e.g. cancer: cachexia), genetic diseases that directly affect the muscle (e.g. Duchenne and Becker Muscular Dystrophies (DMD, BMD), and Limb-girdle muscular dystrophy (LGMD)).

In this review, we briefly describe the roles of macrophages in skeletal muscle regeneration, then turn our focus to their roles in muscular dystrophies (MD) and how current treatments act on their functions.

MACROPHAGES, DEFINITION AND FUNCTION IN SKELETAL MUSCLE REMODELING

To study muscle regeneration and inflammation, injury models that make use of toxins (notexin, cardiotoxin), chemicals (barium chloride) and mechanical trauma (crush, freezing, ischemia, laceration) are used to stimulate a response. Depending on the type of damage the kinetics of the inflammatory response will vary, however the overall regenerative process of the tissue remains the same (for more information about injury models, please read [18]).

The study of macrophages *in vitro* is usually conducted using bone marrow-derived macrophages (BMDM). Macrophage polarization *in vitro* is required to induce the secretion of specific cytokines

Table 1
Monocyte/Macrophage populations

	Population	Markers	Functions	References
Blood circulating monocytes	Ly-6C+	Ly6C+, CCR2+, CD11blow, F480low, CX3CR1low	- Patrol blood system and infiltrate tissues	[38, 39]
	Ly-6C-	Ly6C-, CCR2-, CD11b+, F480+, CX3CR1+	- Patrol blood system	
Tissue resident Macrophages	Embryonic liver-derived	Ly6C-CCR2-F4/80hi CD11blow Lyve1high	- unknown	[26, 31]
	Adult bone marrow-derived	Ly6C-CCR2-F4/80low CD11bhigh Lyve1low	- unknown	
Acute damage	Ly-6C+	Ly6C+, CCR2+, CD11blow, F480low, CX3CR1low, CD11c+	- Activate MuSC proliferation. - Phagocyte debris and dead myofibers - Kill FAPs	[17, 52, 56, 59]
	Ly-6C-	Ly6C-, CCR2-, CD11b+, F480+, CX3CR1+, CD11c+	- Support myogenesis and myofiber growth - Support FAP survival	
Chronic damage	Ly-6Chigh	Ly6Chi, CCR2+, CD11blow, F480low, CX3CR1low, CD11c+	- Activate MuSC proliferation. - Kill FAPs	[56, 59]
	Ly-6C-	Ly6C-, CCR2-, CD11b+, F480+, CX3CR1+, CD11c+	- Activate MuSC proliferation. - Kill FAPs	

associated to their functions such as phagocytosis of apoptotic cells, cell growth and tissue repair promotion, or fighting bacterial infections. Like T cells, macrophages can acquire two main inflammatory profiles: classically activated M1 (related to type 1 inflammation – Th1) and alternatively activated M2 (related to Type 2 inflammation – Th2). The Th1/Th2 paradigm in macrophage has been extensively discussed and we encourage reading the following reviews [19, 20]. BMDM are stimulated with IFNγ, IL-4/IL-13, or IL-10, to respectively mimic either the pro-inflammatory/classically activated state "M1", the alternative activation state "M2a", or the anti-inflammatory state "M2c" [21]. Other M2 states have been proposed, such as M2b (immune complex activation [22]) and M2d (TLR antagonist [23, 24]), but are not relevant in muscle biology. Moreover, Lipopolysaccharide (LPS) alone or together with IFNγ, can be used to induce a stronger pro-inflammatory activation state. However, in the case of tissue regeneration and sterile inflammation, the use of LPS and of these *in vitro* methods in general might push macrophages into state not representative of that found *in vivo*.

The understudied tissue-resident macrophages

Skeletal muscle resident macrophages are quiescent cells, occupying space within the connective tissue that surrounds myofibers and in close proximity to blood vessels [25, 26]. As in other tissues, distinct subsets of resident macrophages arise from either developmental origins (yolk sac-, aorta-gonad-mesonephros- (AGM), or liver-derived hematopoiesis) or from the adult bone marrow (BM) [27]. It has been shown that tissue-resident macrophages acquire tissue specific functions. For example, Kupffer cells (liver) play a crucial role in the clearance of blood toxins, and alveolar macrophages (lung) will actively clean pathogens and microorganisms from the airways [28–30]. In skeletal muscle, so far the only proposed role of tissue resident macrophages is the attraction of circulating blood monocytes to the site of damage [26]. Yet, tissue resident macrophages are still heterogeneous, with a sub-type expressing stress-response genes such as *Klf2* or *Fos* [31].

It has been established that tissue-resident macrophages derive from primitive hematopoiesis in the liver and definitive hematopoiesis in the BM [32, 33]. As of now, the muscle research community lacks a specific marker to distinguish skeletal muscle resident macrophages from infiltrating monocytes. However, it seems that a proportion of resident macrophages are embryonically derived, while another subpopulation is maintained by blood-derived monocytes [31, 32, 34]. The embryonic-derived tissue-resident macrophages are Ly-6C^{-}CCR2^{-} F4/80^{hi}CD11b^{low}, while the BM-derived tissue-resident macrophages are Ly-6C^{-}CCR2^{-}F4/80^{low} CD11b^{high} [32] (Table 1). More recently, the marker

Lyve1 has been added to the list of the markers for tissue resident macrophages [31, 35]. Wang et al., also suggest that Lyve1 expression (high versus low) could differentiate between the embryonic-derived ($Lyve1^{low}$) and the blood-derived ($Lyve1^{high}$) tissue resident macrophages (Table 1). [31] In the heart, tissue-resident macrophages can be distinguished by expression of the marker T cell Immunoglobulin and Mucin Domain containing 4 (TIMD4). $TIMD4^+$ macrophages are able to locally self-renew, while $TIMD4^-$ cells are replaced by blood-derived cells [36]. Moreover, cardiac resident macrophages seem to have very specific functions depending on their location. For example, resident macrophages present in the atrioventricular nodes are required to maintain cardiac contraction via the formation of gap junctions with cardiomyocytes [37]. The regulation, self-renewal, and function of skeletal muscle-resident macrophages at homeostasis and during regeneration is not yet understood. The characterization (ontology and specific markers) of this population should be definitively one of the focus points for better understanding of their function at steady state, during tissue remodeling (mild or acute damage), ageing, and in disease [2, 30].

Infiltrating monocytes and their function as macrophages

Circulating blood-monocyte infiltration

In blood, monocytes separate into two subsets [38]. The first, which is $CCR2^-Ly\text{-}6C^-CX3CR1^{high}$ $CD11b^+F4/80^+$ functions to patrol the vasculature in search of pathogens [39] (Table 1). The other subset is $CCR2^+Ly\text{-}6C^+CX3CR1^{low}CD11b^{low}F4/80^{low}$ and homes to damaged tissues primarily through the CCL2(MCP1)-CCR2 axis [40–44]. This cytokine-receptor interaction was one of the first described to induce monocytes infiltration into tissues after damage and has been observed as indispensable in liver, heart, and skeletal muscle [43, 45–47]. Since then, other chemokines have been described, such as: RANTES/CCL5, MIP3/CCL3, MIP4/CCL4, MCP-3/CCL7, MCP-4/CCL8 [48]. Sources of these chemoattractant can vary by tissue and context. For example, deletion of tissue resident FAPs using the PDGFRα-CRE:DTA mouse model induced a strong reduction in infiltrating $CD45^+$ cells after damage in skeletal muscle [49]. While this has not been backed up with migration assays *in vitro*, FAPs are known to produce and secrete MCP-1 and CSF1, which are known chemoattractant for leucocytes [50]. Alternatively, cells such as SCs and myofibers have also been shown to attract monocytes/macrophages [51, 52]. Currently, only $CCR2^+Ly\text{-}6C^+CX3CR1^{low}$ $CD11b^{low}F4/80^{low}$ monocytes are known to infiltrate damaged muscle [11, 17, 53, 54]. One of the best pieces of supportive evidence comes from the use of the Nur77-KO mouse model. Nur77 (also known as NR4A1) is an orphan transcription factor involved in cell proliferation. Depletion of Nur-77 induces a block in S phase, leading to apoptosis of $Ly6C^-$ MO in the bone marrow [55]. Interestingly, while Nur77-KO animals lack the $Ly\text{-}6C^-$ blood-circulating monocyte population, $Ly\text{-}6C^-$ macrophages are found in the muscle after acute damage in similar numbers to control littermates [53].

Blood-derived macrophage functions

The infiltration of monocytes and their differentiation into macrophages is essential for proper skeletal muscle regeneration [11, 16, 17, 40, 56–58]. Once extravasated, blood monocytes differentiate into inflammatory macrophages and lose CCR2 expression. Of note, this differentiation step is not fully understood yet and may be independent of the process leading to the appearance of alternatively activated and pro-regenerative macrophages (skewing), which is associated with the downregulation of Ly-6C [11, 53] (Fig. 1). $Ly\text{-}6C^+$ and $Ly\text{-}6C^-$ macrophages are observed in a temporally precise sequence and are efficiently coordinated for skeletal muscle regeneration.

Pro-inflammatory macrophages ($Ly\text{-}6C^+F4/80^+$ $CD11b^+CX3CR1^{low}$) secrete cytokines and growth factors that support SC proliferation but induce death in FAPs [52, 56, 59]. Following the clearance of debris, dead cells, and necrotic myofibers, macrophages slowly activate a program that skews them towards a pro-regenerative phenotype ($Ly\text{-}6C^-F4/80^+CD11b^+CX3CR1^{high}$), which supports myogenic cell differentiation and fusion [17, 52, 60–62], and FAP survival [56, 59]. Knockout murine models have demonstrated the importance of this phenotypical skewing for efficient muscle regeneration and identified several actors guiding this process, including AMP-activated Kinase (AMPK), Mitogen-activated protein kinase (MAPK) phosphatase 1 (MKP-1), CCAAT-enhancer-binding proteins (C/EBPβ), and Nuclear Factor I X (Nfix) [17, 63–65]. To note, in addition to Ly-6C, F4/80 and CD 11b, other markers can also be used to distinguish pro-inflammatory macrophages from the pro-regenerative type and are highlighted in Fig. 1 and

Table 2
Markers for ma macrophages involved in skeletal muscle regeneration

Markers	Population	Functions	References
CCR2	Infiltrating monocyte	CCL2 receptor	[15, 40–42, 45, 58]
iNOS	Pro-Inflammatory (Ly-6C+)	Transform Arginine in Nitric Oxid	[21]
Cox-2	Pro-Inflammatory (Ly-6C+)	Catalyzes the conversion of arachidonic acid to prostaglandins	[16, 21]
CD163	Pro-regenerative (Ly-6C-)	Scavenger Receptor	[11, 17]
Arginase 1	Pro-regenerative (Ly-6C-)	Transform Arginine in Ornithine	[21, 231]
Fizz1/RELMa	Pro-regenerative (Ly-6C-)	Pro-fibrotic secreted cytokine	[21, 231]
CD206/MRC-1	Pro-regenerative (Ly-6C-)	Mannose Receptor, specific function unknown	[11, 17, 21]

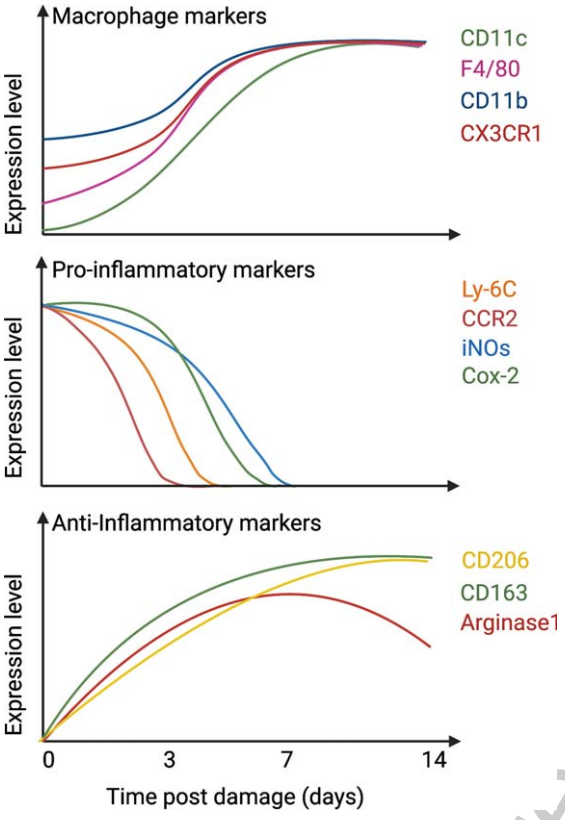

Fig. 1. Temporal expression of macrophage and inflammatory markers. After damage, infiltrated monocytes differentiate into macrophages, up-regulate CD11c, CD11b, F4/80, and CX3CR1 (top graph) and express pro-inflammatory markers such as Ly-6C, CCR2, iNOs, and Cox-2 (middle graph). After 1-2 days in the tissue, they downregulate pro-inflammatory markers and start to express anti-inflammatory proteins such as CD206, CD163, and Arginase 1 (bottom graph).

Table 2. Through this process, macrophages are sensitive to and secrete various cytokines required for efficient skeletal muscle regeneration, briefly described here (Fig. 2):

Annexins are known for their role in resolving inflammation by inducing neutrophil apoptosis and activating monocyte phagocytosis. Specifically, Annexin A1 has long been studied *in vitro* in models of tumor growth and arthritis [66, 67]. While Annexin A1 is expressed by a number of cell types (FAPs, SCs, and macrophages), its receptor FFPR2 is expressed only by macrophages [68] and activates AMPK, promoting phagocytosis and inducing macrophage skewing toward a pro-regenerative phenotype [17, 61].

IGF-1 (Insulin-like Growth Factor 1) has been thoroughly described for its anabolic effect on skeletal muscle [69–72]. However, only more recently has it been shown to also be macrophage-derived. Indeed, deletion of IGF-1 in leukocytes using the Lysozyme^{CRE} mice induces a delay in muscle regeneration due to a defect in pro-regenerative phenotype switching [73].

Interferon gamma (IFNγ) is a pleiotropic cytokine produced by various cell types and seems to have a dual role on myogenesis. So far, IFNγ has been described as expressed by myogenic cells and NK cells, but not in macrophages [74,75]. However, macrophages are sensitive to IFNγ's effects as a pro-inflammatory cytokine. Consequently, IFNγ signaling has to be quickly downregulated by regulatory T cells (Treg: FoxP3^{+}CD4 T cells) in order for macrophages to resolve the inflammation and skew toward a pro-regenerative profile [75]. Interestingly, IFNγ has been shown to have pro-myogenic functions as IFNγ-KO mice display impaired skeletal muscle regeneration [74]. However, over-stimulating human myoblasts with IFNγ *in vitro* also leads to a defect in myogenesis, independently of myoblast apoptosis [76]. Moreover, IFNγ has been shown to antagonize TGFβ signaling in FAP-like cells. Indeed, IFNγ treatment reduces fibrosis in skeletal muscle after laceration injury, which is probably due to FAP apoptosis [77, 78].

Interleukin 1 beta (IL-1β) is part of the IL-1 superfamily (with IL-1α and IL-33) and is mostly expressed by pro-inflammatory macrophages immediately after damage [11, 79]. IL-1β receptors (IL-1R1 and IL-1R2) are not only expressed by macrophages but also in SCs and FAPs [68]. *In vitro*,

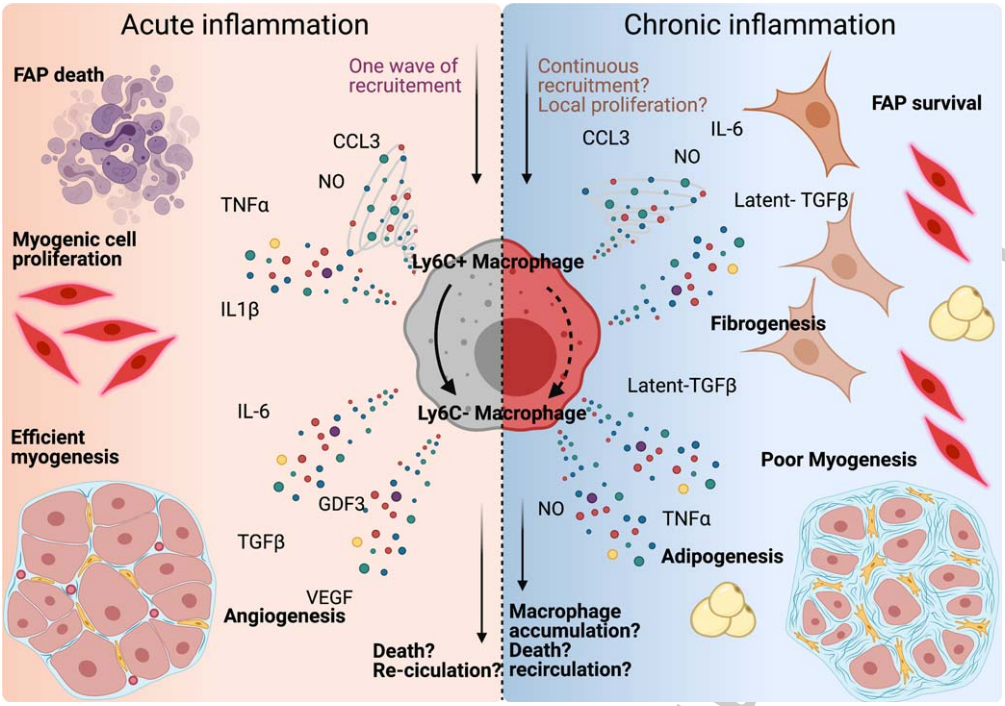

Fig. 2. Macrophages orchestrate muscle-resident cell behaviour during tissue repair. Left panel: after injury, Ly-6C+ macrophages secrete CCL3, NO, TNFa and IL-1b, clear fibro-adipogenic progenitors (FAPs) from the tissue, and support myogenic cell proliferation. Once skewed to an anti-inflammatory profile, macrophage produce IL-6, GDF3, TGFb, and VEGF, which participate into the support of the myogenic program and myofiber growth. Macrophage content within the tissue returns to basal around 2 weeks after damage by either re-circulation or local apoptosis. Right panel: in case of repeated trauma, the number of macrophages present in the tissue rises, which could be due to continuous infiltration, or to local proliferation. Both Ly-6C+ and Ly-6C– macrophages are present within the tissue, which causes the accumulation of both pro- and anti-inflammatory cytokines in the damaged area. FAPs are activated, differentiate into fibroblast and adipocytes and myogenesis is delayed.

stimulation of C2C12 cells or primary myoblasts with IL-1β induces cell proliferation [79]. This effect is probably due to the activation of NFκB signaling, which is key for cell proliferation and survival. Of note, adding IL-1β blocking antibody in human myogenic cell culture with macrophage-conditioned medium induced myotube formation [52]. This suggests that IL-1β also has an anti-myogenic role, protecting from early differentiation.

Interleukin 10 (IL-10) is required for macrophages to acquire a pro-regenerative function [80, 81]. However, early delivery of IL-10 will prematurely induce macrophage skewing, delaying skeletal muscle regeneration [80]. This confirms that macrophage skewing needs to be precisely temporally regulated as faster resolution of inflammation does not always induce better recovery. This is supported by observations following the use of ice or anti-inflammatory compounds such as nonsteroidal anti-inflammatory drugs (NSAIDs) [2, 82]. Indeed, studies have reported that the use of NSAIDs 1) did not reduce soreness after exercise, 2) negatively affected SC fate, and 3) reduced muscle protein synthesis post exercise. However, in > 65-year-old adults, as well as in old rats, NSAIDs help in the gain of muscle mass. Altogether, mouse, rat, and human studies report conflicted results in the use of NSAIDs, thus their use should be consciously done [83].

Interleukin 6 (IL-6) production has been observed in almost all cells present in skeletal muscle (FAPs, endothelial cells, smooth muscle cells, myofibers, and various immune cells such as eosinophils and macrophages). Like TGFβ and TNFα, IL-6 has both pro and anti-inflammatory functions [84], making understanding its role in regeneration complicated. To note, IL-6 is produced at a high level after exercise, but the source seems to be myofibers rather than infiltrating immune cells [85]. However, the concentration of IL-6 peaks at day 6 after acute injury, and delivery of IL-6 *in vitro* has interestingly no effect on proliferation of the myogenic cell line C2C12 [79]. However, as observed with IL-1β, blocking IL-6

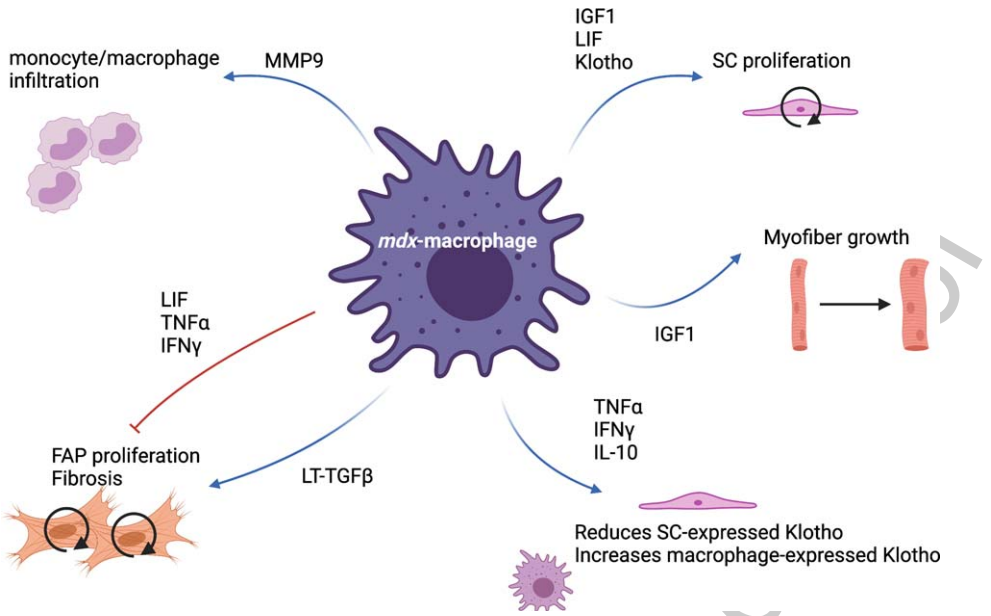

Fig. 3. mdx-macrophage specific functions. Via the production of many cytokines and proteins, macrophages present in mdx muscle are able to simultaneously stimulate and inhibit various cellular processes such as monocyte infiltration, satellite cell (SC) proliferation, myofiber growth, fibro-adipogenic progenitor (FAP) proliferation and differentiation.

in human macrophage conditioned medium induces myotube formation *in vitro*, suggesting an anti-myogenic function of macrophage-derived IL-6 [52].

Interleukin 13 (IL-13) and Interleukin 4 (IL-4) are type 2 cytokines usually secreted by eosinophils, T cells, and Innate Lymphoid Cells (ILCs). Both have been described to induce macrophage skewing [86, 87]. To note, IL-4 treated FAPs support SC differentiation *in vitro* [86]. Plus, the IL-4/IL-13 axis also blocks FAP differentiation into adipocytes, allowing efficient regeneration [86]. In adipose tissue, it has been suggested that the IL-4/IL-13 axis is required for tissue resident mesenchymal stromal cells (FAP-like cells) to produce CCL11 (Eotaxin1) and attract eosinophils to maintain a type 2 inflammatory environment [87–89]. This chain of events has not been demonstrated in skeletal muscle, but represents a potential mechanism for eosinophil recruitment and the resolution of inflammation.

Nicotinamide phosphoribosyltransferase (NAMPT), also known as Visfatin or PBEF) is secreted by a specific population of macrophages during regeneration and acts on SCs via CCR5 to induce their proliferation [62]. In a zebrafish model, NAMPT+ macrophages appear to assume a pro-regenerative role similar to that observed in mouse and human, where they appear 2 days after injury and express *arg2*, mmp13, and *mmp9* [62].

Transforming growth factor beta (TGFβ) is a complex cytokine known to affect the behavior of most cells through disparate signaling pathways. The TGFβ superfamily is composed of 50 members, with TGFβ1 and myostatin being the most highly expressed in skeletal muscle [90]. One of the best known functions of TGFβ is its potent effect on fibrogenesis [8, 9, 91]. For example, TGFβ induces FAP survival (counteracting the IFNγ/TNFα pro-apoptotic effect) and differentiation into myofibroblast [59]. TGFβ is also known for its inhibitory effect on myogenesis [92–95]. As TGFβ ligands (TGFβ1, 2, and 3) are secreted by multiple sources, it is difficult to discern paracrine and autocrine function. Interestingly, macrophage-secreted GDF3, which is a ligand of the TGFβ superfamily, has been shown to promote SC commitment to myogenesis [60]. Lastly, TGFβ is known to be secreted in a non-active form (called latent TGFβ), which is sequestered in the ECM in complex with latent TGFβ binding proteins (LTBP) [96]. Thus, its activity and action on SCs and FAPs can temporally differ from its secretion.

Tumor Necrosis Factor alpha (TNFα). TNFα is another complex cytokine, which can have different effects that are dependent upon its concentration. Interestingly, when secreted at high concentrations by inflammatory macrophages, it has anti-myogenic

properties. However, when TNFα is secreted at low levels by anti-inflammatory macrophages, it instead functions as a pro-myogenic cytokine [52]. This differential effect is probably compounded by a globally changing cytokine milieu in the regenerative process. It would be interesting to study if the dual function of TNFα is only due to its concentration, or to its cooperation with other cytokines (e.g. IL-1β).

Vascular endothelial growth factor (VEGF). While VEGF is mostly known for its pro-angiogenic functions, its effects on myogenic cell behavior have recently been explored [97, 98]. Indeed, inhibition of VEGF induces myotube formation *in vitro*, suggesting an anti-myogenic function of macrophagic VEGF [52]. However, Verma et al., showed that SC-derived VEGFA helps to maintaining the niche micro-environment and encourages their own quiescence [98]. Interestingly, quiescent SCs do not appear to express detectable levels of VEGF receptors (*Kdr* (VGFR2) or *Flt1* (VGFR1)). Thus, further studies into how VEGFA acts on SC behavior is warranted.

HISTOPATHOLOGY AND ANIMAL MODELS OF MUSCULAR DYSTROPHIES

MDs are a heterogeneous group of inherited disorders characterized by progressive wasting and weakness of muscle tissue that compromises patient mobility, leading to wheelchair dependency. In severe cases, patients with MDs die prematurely due to respiratory and cardiac failure [99]. Many MDs are caused by mutations in the genes coding for proteins of the dystrophin-glycoprotein complex (DGC) or required for the correct assembly of the DGC. The main components of this multiprotein complex are dystrophin and sarcoglycan subunits. Structurally, the DGC links the F-actin cytoskeleton of myofibers to the ECM. The absence of even one protein of the DGC often causes the disassembly of the entire complex, causing sarcolemmal (the myofiber plasma membrane) fragility and leading to myofiber damage and necrosis that is aggravated by contractile activity [100–102]. Damaged myofibers are repaired or replaced by SCs, but as they share the same genetic mutation, the newly formed myofibers are destined for the same degenerative fate. Consequently, muscle tissue enters into a continuous cycle of degeneration and regeneration that results in chronic inflammation and substitution of contractile muscle tissue with adipose and fibrotic tissue. At the histological level, dystrophic muscle is characterized by necrotic myofibers, desynchronized centrally-nucleated regenerative myofibers, immune cell infiltration, and the presence of fatty/fibrotic lesions in place of muscle tissue [103].

The most common MD is the Duchenne muscular dystrophy (DMD), an X-linked autosomal recessive disease caused by a mutation in the ~2.4 Mb dystrophin gene that results in complete loss of the dystrophin protein and affects approximately 1 in 3,500–6,000 boys [104, 105]. The first signs of disease are usually observed around 2 to 3 years of age, followed by progressive muscle wasting, gradually leading to wheelchair use and eventual death caused by respiratory and cardiac complications [99, 106, 107]. In DMD 60% of dystrophin mutations are large insertions or deletions that lead to frameshift errors downstream, whereas approximately 40% are point mutations or small frameshift rearrangements [108]. Becker-type muscular dystrophy (BMD) is also caused by a mutation to the dystrophin gene, but one that permits the synthesis of an internally truncated and partially functional protein, leading to a milder phenotype in affected boys [99]. MDs are particularly difficult to treat due to the post-mitotic nature of cardiac and skeletal muscle, as well as the abundancy of muscle tissue across the body. Different animal models are used to understand the development of the dystrophic disease as well as molecular and cellular pathways involved in this process [109]. Here are some of the main mouse models:

***mdx* mice:** the mouse model for DMD is called *mdx* and is the main animal model used to study MDs. The *mdx* mutant came from a spontaneous mutation in a colony of C57BL/10ScSn mice, first reported in 1984 by Bulfield. Myofiber necrosis appears in the limbs of *mdx* mice at 3 weeks of age and a first peak of inflammation and necrosis occurs at 4 weeks. From this point forward, the muscle enters a cycle of degeneration and regeneration. At 8 weeks of age, 80% of myofibers are centrally-nucleated and myofiber size is highly heterogeneous [110–113]. Due to the lack of the DCG, *mdx* myofibers are fragile, and damage can be easily induced through muscle contraction (similar to eccentric exercise). Unlike human DMD muscles that are progressively replaced by fat and fibrosis, limb muscles of young *mdx* mice develop only mild fibrosis and no fat infiltration, suggesting that *mdx* muscles have a higher capacity for regeneration compared to human dystrophic muscle. At 12 months of life, *mdx* muscle is still regenerative [111, 112] and it is not until 18 and 24 months of age that fibrosis and adipose infiltration are observed, respectively

[110]. The only *mdx* muscle that faithfully recapitulates human DMD progression is the diaphragm. In this tissue, the first histopathological muscle lesions appear at 1 month of life and over time myofibers are replaced by fibrotic tissue [114, 115]. Thus, while human DMD patients and *mdx* mice both lack dystrophin, mice do not progress to the same level of pathophysiological severity, which limits their use for modelling human DMD. Since the mdx mouse model does not adhere to the general symptoms of the human disease, some variations of the mouse model have been generated in order to better mimic DMD pathology:

Micro-damage: a mechanical strategy entailing repeated daily microneedle stabs, inducing fibrosis and myofiber size heterogeneity 1-week post-injury in the tibialis anterior of *mdx* mice. Asynchronous regeneration is linked to appearance of fibrotic tissue and failed regeneration [116, 117].

***mdx:*utrn+/– mouse model:** a genetic strategy used to create *mdx* models that develop fibrosis earlier. It is based on the hypothesis that some structural proteins, such as UTROPHIN could compensate the lack of dystrophin in *mdx* mice. Double knockout *mdx*:utrn$^{-/-}$ mice show severe progressive MD, leading to premature death, although haploinsufficiency of the *utrn* gene is enough to induce early fibrosis in limb muscles in *mdx* mice [118, 119].

D2-*mdx*: another hypothesis advanced to explain histological differences between *mdx* mice and DMD muscle was a higher regenerative capacity inherent to the murine genetic background. In fact, after several rounds of cardiotoxin injury, C57BL10 mouse strain muscles regenerate efficiently while muscles of DBA/2 mice display fibrosis and fat infiltration [120]. This impaired regeneration is due to a decrease in SC proliferation, leading to a decrease in myogenic cells available for fusion and consequently smaller regenerated myofibers. The most commonly used *mdx* murine model is on the C57BL10 background, while *mdx* mice backcrossed with the DBA/2 background (or D2-*mdx*) exhibit more rapidly progressing dystrophic pathology [120–122]. D2-*mdx* display muscular atrophy and an increase in fibrotic area in tibialis anterior, gastrocnemius and quadriceps muscles compared to *mdx* mice [120, 121, 123].

***mdx*$^{\text{betageo}}$**: Recently, Young et al., generated a model of *mdx* with complete loss of dystrophin by introducing a disruption in the reading frame downstream of exon 63: *mdx*$^{\text{betageo}}$ [124]. These dystrophic mice do not express the full length Dp427 isoform of dystrophin but do express the truncated Dp71 isoform. The total absence of the long dystrophin isoform induces a decrease in myofiber size, and an increase of fibrosis and calcification in the tibialis anterior. Calcification was also observed in other limb muscles and in the heart of *mdx*$^{\text{betageo}}$ mice. Interestingly, high numbers of macrophages were found around calcified myofibers [124]. This model is often compared to the DMD-null mouse model that lack the full length of the dystrophin protein (both Dp71 and DP427 isoforms) [125, 126].

α-sarcoglycan deficiency: the mouse model for α-sarcoglycan deficiency (sgca$^{(-/-)}$ mouse) was designed to model an existing sarcoglycan human mutation, found in patients of LGMD type 2D/R3 [132]. Necrotic myofibers are observed at 3 weeks of age and more than 70% of myofibers are centrally-nucleated in limb muscles and diaphragm at 8 weeks. The sgca$^{(-/-)}$ mouse exhibits a decrease in muscle function and, importantly, fibrosis occurs earlier in limb muscles and the diaphragm [132–134]. So even while the genetic origin of the disease is different between *mdx* and sgca$^{(-/-)}$ mice, the development of DM pathology in the latter more closely captures that observed in human MDs.

In *mdx*, sgca$^{(-/-)}$, GRMD, and DMD, a highly inflammatory environment (in terms of cell infiltration and cytokine detection) is observed compared to WT, non-damaged muscles [111, 115, 128, 134–137]. While macrophages are required and beneficial for efficient muscle regeneration after acute injury (see chapter above), chronic inflammation leads to tissue damage. In the case of skeletal muscle, the deleterious effect of macrophages during periods of chronic damage is hypothesized to be attributed to: 1) a sustained inflammatory environment that promotes muscle tissue damage, and 2) fibrosis induced by abnormal persistence of wound-healing macrophages. Indeed, in the last decades, many studies thoroughly investigated the phenotype and functions of macrophages in MDs.

ROLE OF MACROPHAGES IN CHRONIC INJURY: FRIENDS OR FOES?

Muscle regeneration following acute injury can be impaired by genetically or pharmacologically affecting macrophage's capacity to infiltrate the tissue [11, 57, 58, 138–140]. However, in dystrophic muscle the decreased inflammation associated with modifying macrophage's infiltrative capacity often correlates with improved tissue functionality. In both human

Table 3
Depletion of monocyte/macrophage in mdx mouse model

Mouse model	Result	References
mdx injected with anti-F4/80 antibody	- Improved soleus muscle at 1- and 4-weeks of age	[141]
mdx:TLR4-KO	- Improved TA and diaphragm muscle histopathology at 6- and 12-weeks of age	[142]
mdx:CCR2-KO	- Improved diaphragm muscle histopathology and function at 6- and 12-weeks of age	[143, 145]
	- No improvement of diaphragm muscle histopathology and function at 6-months of age	
mdx injected with CCR2/CCR5 antagonist	- Decreased macrophage infiltration at 2- and 6-weeks of age with no effect on diaphragm muscle histopathology	[144]
	- No changes in macrophage polarization (iNOs/CD206 ratio)	
mdx:CD11b-DTR	- Worsening TA muscle histopathology at 12-weeks of age	[147]

DMD, and mouse models like in sgca$^{(-/-)}$ and *mdx*, the number of macrophages correlate with expression of fibrotic markers [56].

Depletion of blood-circulating monocytes in young *mdx* mice using an anti-F4/80 antibody reduces the number of damaged myofibers, presumably because it delays the peak of inflammation that normally occurs in *mdx* mice at early time point [141] (Table 3). Preventing macrophage infiltration by deleting the TLR4 receptor also attenuates MD progression in 6 and 12-week-old *mdx* mice by limiting muscle damage and fibrosis, while also limiting loss of force [142] (Table 3). Likewise, deleting the CCR2 receptor in *mdx* mice (*mdx*-CCR2$^{-/-}$) decreases macrophage infiltration in the diaphragm at 6 weeks of life. Interestingly, at 12 weeks of age, *mdx*-CCR2$^{-/-}$ mice have the same number of intramuscular macrophages compared to *mdx* control mice, suggesting a role for tissue-resident macrophages in disease progression. Furthermore, there are more CD206+ (pro-regenerative) macrophages and fewer iNOS+ (pro-inflammatory) macrophages in the diaphragm of *mdx*-CCR2$^{-/-}$ mice at 6 weeks of age. Again, this may be due to contribution of tissue-resident macrophages, which may not be able to efficiently acquire a pro-inflammatory phenotype. No difference in terms of histopathology is observed between *mdx* and *mdx*-CCR2$^{-/-}$ mice at 6 weeks of age, but at 12 weeks of age a decrease in the number of necrotic myofibers and area of fibrosis is observed in the diaphragm of double mutant mice. Functional improvement in the diaphragm is observed at both 6 weeks and 12 weeks of age [143] (Table 3).

Consistent with these results, the use of a pharmacological antagonist of CCR2/CCR5 receptor (CVC or cenicriviroc) in *mdx* mice from 2- to 6-weeks of age decreased macrophage infiltration of the diaphragm in treated mice without any effect on myofiber necrosis and fibrosis. However, no changes in the ratio of CD206+ or iNOS+ macrophage were observed [144] (Table 3). Analysis of *mdx* mouse pathology at 1 year shows that these animals approach a similar disease state to that of human DMD patients (force, regenerative capacity, central nuclei, muscle hypertrophy, and myofiber branching). Similarly, the early improvements observed in the diaphragm and quadriceps of *mdx*-CCR2$^{-/-}$ is lost by 6 months of age in these animals [145]. As in the study by Mojumbar et al., the diminution of macrophage infiltration at an early time point (4 weeks of age) disappears by 14 weeks and 6 months [143, 145], It remains unclear whether the regression in histological and functional improvement is due to re-infiltration of macrophages or from an effect on their inflammatory profile and function.

When *mdx* mice are crossed with a urokinase plasminogen activator (uPA) deficiency model the progeny exhibit an increase in the degenerative muscle phenotype, including increased fibrosis and decreased muscle function. This phenotype also correlates with a decrease in macrophage infiltration in the dystrophic muscle [146] (Table 3). Interestingly, the transplantation of WT-BM into the *mdx*-uPA$^{-/-}$ mice increased macrophage infiltration in dystrophic muscle and reversed the deleterious effect of uPA knockout, suggesting a pro-regenerative role of infiltrating, uPA-expressing macrophages within the dystrophic muscle. Of note, muscular regeneration after acute injury is also observed in uPA$^{-/-}$ mice and is rescued by WT BM transplantation as well [146]. Another study demonstrated that the depletion of macrophages in *mdx* mice (using a mouse model expressing the Diphteria Toxin Receptor under the CD11b promotor) compromises muscle regeneration at 12 weeks of age by promoting adipogenic fate in SCs [147].

In conclusion, the functional phenotype of macrophages and more specifically, their trophic function toward other cells within the muscle, impacts disease progression more than the overall number of macrophages present in dystrophic muscle, and this parameter should be taken in consideration when analyzing muscle histopathology (Table 3).

Trophic functions of macrophages toward muscle homeostasis in mdx mice

During healthy muscle regeneration, two functionally distinct populations of macrophages are observed in a temporally precise sequence. Together, these pro-inflammatory Ly-6C^{+} and pro-regenerative Ly-6C^{-} macrophages efficiently coordinate to help heal the damage and return the affected muscle tissue back to homeostasis. However, the situation becomes more complex in dystrophic muscles, where gauging the functional status of the macrophages within the affected tissue using simple markers such as Ly-6C may be less reliable (Fig. 1 and Tables 1 and 2). In fact, a recent work suggests that this method fails to accurately capture the nuances of macrophage transcriptional status within these situations [56]. During muscle regeneration, macrophages simultaneously express pro- and anti-inflammatory programs, and their function is ultimately determined by the balance of these programs [17, 54, 56] (Fig. 1 and Table 1). Gene expression analyses of Ly-6C^{+} and Ly-6C^{-} macrophages sorted from non-fibrotic and fibrotic dystrophic muscle (*mdx* and sgca$^{(-/-)}$ mice) show that the canonically pro-regenerative Ly-6C^{-} population actually express high levels of pro-inflammatory markers, suggesting the presence of a mixed-function population of macrophages within dystrophic muscle [56]. Indeed, nearly 50% of macrophages present in *mdx* mice express both TNFα and TGFβ [59]. Future studies that incorporate single cell and spatial RNA-sequencing technologies allow us to better understand macrophage polarization and function in dystrophic muscles and particularly, how they interact with other cell types. Nevertheless, numerous studies have helped to identify general functions of macrophages in chronic muscle injury.

Genetic ablation of IFNγ in *mdx* mice (*mdx*:IFNγ-KO) does not affect macrophage infiltration in 4 and 12 month-old mice, but does lead to a decrease in iNOS production and reduced damaged myofibers in hindlimb muscles [148]. The loss of IFNγ also increased *MyoD* and *Myogenin* expression, which are markers of proliferating and differentiating myoblasts whose expression is linked with regeneration. Indeed, *mdx*:IFNγ-KO mice have more centrally-nucleated myofibers and exhibit improved muscle function, suggesting enhanced tissue repair [148].

Deletion of iNOS in *mdx* mice (*mdx*:iNOS-KO) has no effect on macrophage infiltration, but decreases myofiber lysis [149]. In DMD and *mdx* muscles, a local increase in TNFα has been observed, and leads to the activation of NFκB signaling [150, 151]. Consistently, NFκB activity increases in diaphragm, gastrocnemius and tibialis anterior of 5-week-old *mdx* mice compared to WT mice. In *mdx* mice, NFκB is present in nuclei of both regenerating myofibers and immune cells.

Macrophage-specific deletion of NFκB in *mdx* mice decreases the number of necrotic myofibers and results in reduced expression of TNFα and IL-1β in 4-week-old animals. Interestingly, the specific deletion of NFκB in myofibers stimulates muscle regeneration and leads to an increase in embryonic myosin heavy chain positive (eMHC^{+}) myofibers. Furthermore, an increase in cells expressing Pax7 and MyoD is observed in 4-week-old *mdx*-NFκB-KO mice, suggesting a negative role of NFκB on SC behaviour. Finally, pharmacological inhibition of IKK/NFκB pathway reduces muscle necrosis and improves muscle regeneration, supporting the notion that this pathway is active in several cell types present in dystrophic muscle which act synergistically to rescue myopathic progression [151].

Weekly intra-peritoneal injections of TNFα-blocking antibodies in *mdx* mice during the first 90 days of life decrease the number of necrotic myofibers and have a positive effect on treadmill running time. Unfortunately, neither the inflammatory profile, nor possible mechanisms of action were investigated in this study [152]. The use of anti-IL-6 antibody on *mdx*:utrn$^{-/-}$ from 2 to 13 weeks of age, was shown to significantly improve skeletal muscle histopathology by reducing Creatine Kinase (CK) levels, fibrosis deposition, increasing regenerating myofiber size [153]. However, these effects were not seen in the diaphragm. Paradoxically, while IL-6 has been shown to participate to the DMD pathology [154], the inflammatory response, quantified by q-RT-PCR was unchanged [153]. Moreover, Kostek et al. treated *mdx* mice with IL-6 blocking antibodies for 5 weeks with no functional or histological improvements, but rather an increase in their "inflammation score" (quantified by the number of mononuclear cells observed on muscle slides) [155].

In a related recent study, 4-month-old *mdx*:utrn$^{+/-}$ mice were injected intra-peritoneally every 3 days, for 28 days, with blocking antibody against RANKL (receptor activator of nuclear factor NFκB ligand) [156]. While muscle damage and fibrosis were decreased, and associated with an increase in myofiber size, the number of infiltrated macrophages did not change after treatment. Nevertheless, a relative increase of CD206$^+$ macrophages was observed in muscle of *mdx*/utrn$^{+/-}$ mice injected with RANKL blocking antibody [156]. Similarly, the frequency of CD206$^+$ cells increased in muscles of *mdx*:IFNγ-KO mice [148].

Mdx:IL-10-KO mice have elevated numbers of necrotic myofibers and perform poorly in treadmill performance tests at both 4 and 12 weeks of age, when compared to *mdx* mice. These mice have a lower frequency of CD163$^+$ pro-regenerative macrophages in muscle, but quantification of total macrophage infiltration was not performed [81]. Thus, IL-10 secretion seems to be beneficial for dystrophic muscle.

Finally, the beneficial effect of depleting specific pro-inflammatory cytokines or signaling pathways in MD pathology could be due to the fact that dystrophic myofibers are more sensitive to oxidative stress [157, 158].

Overall, the above studies suggest that decreasing macrophage pro-inflammatory signals and pushing them to an anti-inflammatory phenotype could be beneficial for dystrophic muscle [156]. However, most studies have focused on the first few weeks of life in *mdx* mice, which unlike human patients is known to peak in inflammation at 4-weeks of age [141, 159]. Thus, further detailed studies and critical evaluation of these datasets will be required to assess the potential of macrophage modulation as a therapeutic option for MD patients.

The role of macrophages in the formation of fibrosis in muscular dystrophies

At steady state, muscle ECM is a three-dimensional network that represents around 5% of tissue volume. The ECM is primarily composed of collagen type I, with myofibers being surrounded by collagen IV [160]. ECM's function as a structural substrate capable of supporting muscle fibers, blood vessels, and nerves must not eclipse its critical role as a regulator of cell fate. For example, it has been demonstrated that Collagen V and VI are intimately linked to SC quiescence [161, 162]. After muscle injury, ECM is degraded by inflammatory cells, which permits their infiltration into the damaged tissue, while concomitantly facilitating effective migration of SCs [163, 164]. Specifically, the ECM is degraded by matrix metalloproteinases (MMPs), secreted by inflammatory cells and damaged myofibers [165–167]. A transient partially degraded ECM participates in myoblast differentiation and provides a scaffold for regenerative myofibers [168]. As such, the balance between ECM degradation and production is important for efficient regeneration. Fibrosis, which is a characteristic feature of MD pathology, is caused by excessive accumulation of ECM components resulting from ECM over-production, a defect in its degradation, or both [168, 169]. Among the factors that promote ECM remodeling, TGFβ is believed to be the most important. TGFβ not only induces collagen I, connective tissue growth factor (CTGF/CCN2) and fibronectin expression by FAPs/fibroblasts, but also inhibits MMP expression in fibroblasts through SMAD pathway activation [90, 170–172]. While macrophages and SCs do express some collagen proteins, FAPs are the primary source of ECM component secretion [162, 173, 174]. FAPs were identified in 2010 and are defined as SCA-1/PDGFRα^+, CD31/CD45/α7int$^-$ cells. FAPs are located in the skeletal muscle interstitial space and proliferate upon injury [8, 9]. In both *in vitro* and *in vivo* conditions, FAPs are capable of spontaneously differentiating into fibroblasts and adipocytes, but they do not differentiate into myogenic cells [8, 9, 50]. They stimulate myogenic differentiation in SCs, which once differentiated into myofibers, block FAP adipogenic differentiation [8, 175, 176]. In *mdx* mice, the number of PDGFRα^+ cells positively correlates with fibrosis and addition of TGFβ1 to FAPs *in vitro* induces a dose-dependently increase in fibrotic markers (such as collagen I and CTGF [8, 9, 59]), supporting the importance of this factor in fibrosis development. Importantly, the balance between proliferation and apoptosis of FAPs is directly determined by macrophage-derived TGFβ1

The unforgettable TGFβ

During skeletal muscle regeneration, pro-inflammatory macrophages first secrete TNFα, which induces FAP apoptosis bringing their numbers back to pre-damage levels. Next, pro-regenerative macrophages secrete TGFβ to stimulate the survival of remaining FAPs and the production of a regenerative provisional matrix. Proper balance and coordinated expression of these cytokines is thus critical for reestablishment of the ECM [56, 59, 177]

(Fig. 2). For more background on the role of FAPs in muscle homeostasis, we suggest the following reviews [178, 179].

In DMD patients, TGFβ1 is elevated in both blood plasma and muscle, and is correlated with fibrosis [91, 180, 181]. In addition, treatment of WT mice with recombinant TGFβ1 stimulates the expression of collagen I and induces muscle fibrosis independently of injury or disease [182]. It has been demonstrated that asynchronous myofiber damage and regeneration, such as that observed in DMD, directly induces fibrosis through the TGFβ1 pathway [117]. However, the induction of a fibrogenic program in FAPs is not the only way in which TGFβ1 can modulate muscle homeostasis. The binding of TGFβ1 and/or myostatin to their specific cell-surface receptors (TGFBR1/ALK5 and TGFBR2 dimer for TGFβ1; activin receptor types IIA and IIB dimer, or TGFBR1/ALK5 and ALK4 receptor dimer for myostatin) can lead to a decrease in the expression of the muscle fiber hypertrophic factor IGF-1 [93, 183].

TGFβ1 is expressed by multiple different cell types such as FAPs/fibroblasts and endothelial cells, but mainly by macrophages: over 75% of these cells in the *mdx* diaphragm express TGFβ [90, 171, 184]. One important feature of TGFβ1 signaling is that ligand gene expression may not directly lead to downstream signaling activation. This has been demonstrated in animal models where TGFβ expression levels do not correlate with the amount of fibrosis observed [185, 186]. Indeed, secreted TGFβ1 is often found bound to LTBP and is stabilized but kept inactive until LTBP cleavage [187]. Notably, DMD muscles exhibit elevated LTBP4, regulating TGFβ1 availability [188].

Treating *mdx* mice with Nilotinib (which inhibits p38-like kinases downstream of TGFβ) rescues the dystrophic phenotype by decreasing FAP numbers and the associated fibrosis [56, 59]. The dystrophic environment alters the effect of macrophages toward FAPs. Inflammatory macrophages (Ly-6C^{+}CX3CR1^{low}) isolated from fibrotic *mdx* muscle have lost their ability to induce fibroblast apoptosis and instead stimulate collagen I expression [56]. This effect is reversed by anti-TGFβ antibody treatment. Moreover, inflammatory macrophages from fibrotic *mdx* muscle express more LTBP4, which allows for more latent TGFβ1 to be stabilized within the ECM compared to non-fibrotic mdx muscle. In *mdx* muscle, FAPs secrete MMP14 and BMP1 proteases to release TGFβ1 from LTBP4 [56].

Treatment of *mdx* mice with the AMPK activating compound metformin (otherwise used for the treatment of type 2 diabetes) promotes pro-inflammatory to pro-regenerative macrophage phenotype skewing, reduces fibrosis, and improves muscle morphology [56]. In *mdx*:IL-10-KO mice, macrophages increase their expression of pro-inflammatory cytokines and an increase in mature TGFβ and collagen I secretion within muscle tissue is observed [189]. Similarly, fibrinogen (a soluble protein released into the blood in response to stress which accumulates in *mdx* and DMD muscles)-activated BMDMs treated with blocking IL-1β antibodies exhibit a decrease in *Tgfb* gene expression. On the other hand, mdx fibroblasts treated with conditioned medium of fibrinogen activated BMDM increases collagen I (*Col1a1*) expression, and this pro-fibrotic effect can be reverted with TGFβ1 blocking antibody [190]. Together, these studies demonstrate that the atypical pro-inflammatory macrophages found in dystrophic muscle not only act on myofiber damage and repair, but are also capable of directly inducing fibrosis. Thus, in dystrophic conditions, the dysregulation of macrophage phenotype induces a vicious cycle between macrophage LTBP4-TGFβ expression, FAP survival and ECM component expression that leads to fibrosis.

It should be noted that the effects of TGFβ are not limited to FAP/fibroblasts. SCs and endothelial cells also express receptors for this molecule. *In vitro* treatment of SCs and endothelial cells with TGFβ results in decreased myotube and angiotube formation and is associated with an increase in fibrotic gene expression [174]. By using specific endothelial cell and SC tracking mice, this phenomenon has been replicated *in vivo* in 6-month-old *mdx* mice. Specifically, approximately 12% of SCs downregulated a myogenic fate marker (loss of α7-integrin) and showed elevated *Col1a1* and fibronectin extra domain A (*Eda-Fn1*) expression. Similarly, 30% of the original endothelial cell population reduced CD31 expression, with a concomitant increase in *Col1a1* and *Eda-Fn1* [174]. Within dystrophic muscle, infiltrating macrophages also become pro-fibrotic expressing more collagen I and less CD45. These "fibrotic" SCs, endothelial cells and macrophages represent only 1-2% of the total fibrogenic population, and while they may not have a major impact on fibrosis *per se*, they may no longer be capable of participating in muscle and vessels formation. Indeed, myogenic cells, endothelial cells and macrophages must communicate for effective muscle regeneration and it has been observed that *mdx* mice also exhibit impaired vessels formation and functional vascular defects [98, 191, 192].

Other proteins expressed by macrophages that modulate MD progression

Several studies have pointed out additional proteins expressed by macrophages that exacerbate or attenuate muscular dystrophy progression.

MMPs are expressed by macrophages and support cell migration to injured tissue. MMP expression is increased in dystrophic mouse muscle. *mdx*:MMP9-KO mice not only show reduced macrophage infiltration, but also a switch toward a pro-regenerative macrophage phenotype, characterized by an increase in $CD206^{+}$. Moreover, *mdx*::MMP9-KO mouse muscle structure is improved and correlates with a decrease in serum Creatine Kinase levels, which is a marker of myofiber damage [193, 194]. Lastly, depletion of MMP9 increases SC proliferation and improves the engraftment potential of myoblasts in recipient mouse muscle pre-injured with cardiotoxin [194]. However, MMPs also play a beneficial role in the regenerative process that follows acute damage. For example, MMP-10 is expressed by macrophages and endothelial cells in response to injury, and its deletion increases macrophage infiltration, myofiber necrosis and interstitial fibrosis [195]. Batimastat is a broad spectrum MMP inhibitor that acts on MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, and MMP-14 activity by mimicking the site in the collagen substrate that is cleaved by MMPs [196, 197]. Treatment of *mdx* mice with Batimastat increases the levels of DGC protein components, improves muscle structure and force, and reduces the number of damaged myofibers. A decrease in the number of infiltrating macrophages, as well as in fibrosis (decreased *Col3a1* expression and smaller proportion of Picrosirius red stained area) was also observed [198].

IGF-1 induces muscle hypertrophy in mice and rats by promoting SC proliferation and myofiber anabolism [199–201]. Treating *mdx* mice with IGF-1 for 8 weeks, starting from 5-6 weeks of age, improves fatigue resistance in EDL and soleus muscles [202]. Similarly, overexpression of IGF-1 in *mdx* mice induces muscle hypertrophy, increases muscle force and reduces fibrosis [71]. Depleting macrophages in injured muscle significant decreases IGF-1 levels, showing that macrophages are a primary source of IGF-1 within this context. Moreover, deletion of macrophage-specific deletion of IGF-1, as in the $LysM^{CRE}$ mouse strain, induces a defect in pro-regenerative phenotype acquisition and, consequently, delayed muscle regeneration [73]. Overexpression of IGF-1 in myofibers down-regulates the expression of pro-inflammatory cytokines and rapidly stimulates tissue remodeling [203]. This suggests an autocrine effect of IGF-1 on macrophages and a paracrine effect on myogenic cells that appears beneficial within the context of MD.

Leukemia inhibitory factor (LIF) is expressed by myoblasts and macrophages and has a positive effect on SC proliferation [204, 205]. In *mdx* mice, treatment with LIF decreases fibrosis, stimulates muscle regeneration, and increases myofiber size [206, 207]. In 2019, Welc et al., studied the effect of LIF overexpression in macrophages using a transgene controlled by the human CD11b promoter [208]. 1–12-month-old *mdx* mice overexpressing LIF in $CD11b^{+}$ cells showed a reduction in collagen deposits in TA and diaphragm muscle. Bone marrow transplantation (BMT) of transgenic $CD11b^{+}/LIF^{+}$ cells into 1-month-old *mdx* mice decreases macrophage infiltration with a decreased collagen I, IV and V deposit area in tibialis anterior muscles 4 months after transplantation. Interestingly, TGFβ secretion by macrophages is decreased in the presence of LIF, which also decreases FAP numbers. While TGFβ induces CTGF/CCN2 expression in the C2C12 cell line, co-stimulation with LIF abrogates this increase after 24 h in culture. *In vivo*, while SCs from LIF BMT/*mdx* express less ECM related genes, mice do not present any changes in their histopathology [208], consistent with the notion that SCs do not play a major role in collagen deposition.

Klotho is a transmembrane protein that can be cleaved and released as a hormone, or alternatively expressed in a truncated form capable of being secreted [209, 210]. It modulates multiple signaling pathways, including FGFs, IGF1, TGFβ and Wnt [211]. At 2 weeks of age, *mdx* mice display no difference in *Klotho* expression compared to WT mice. As inflammation peaks later during disease progression, *Klotho* expression drops, remaining low until at least 3 months of age. The overexpression of *Klotho* in *mdx* mice decreases fibrosis, increases myofiber size at 24 months, and increases treadmill running time [212]. Interestingly, it has been demonstrated that Klotho signaling is suppressed by TNFα in the kidney [213]. Injection of recombinant Klotho protein decreases pro-inflammatory signals in both kidney and heart tissue [214, 215]. The increase of TNFα and IFNγ observed in *mdx* muscles coincides with a decrease in Klotho [216]. *In vitro* TNFα treatment decreases *Klotho* expression in C2C12 myotubes but activates its expression in macrophages. This phenomenon is also observed in the presence of IL-10 [216]. Of

note, BMT of cells overexpressing Klotho into *mdx* mice hosts increases $Pax7^+$ SCs and myofiber size. Conditioned media co-culture experiments demonstrate that overexpression of Klotho by macrophages directly stimulates proliferation of SCs [216]. Rescue of Klotho expression by macrophages in *mdx* mice improves MD histopathology by acting on myogenic cell proliferation [216].

Osteopontin (OPN) is up-regulated in both DMD patients and in *mdx* mice. OPN, also known as Secreted Phosphoprotein 1 (SSP1) has been described as an upstream activator of NFκB signaling [217, 218]. Depletion of OPN in *mdx* mice does not modify the total number of infiltrated macrophages but skews their phenotype to a less inflammatory profile (from $iNOS^+$ to $CD206^+/CD163^+$). Lack of OPN in *mdx* mice increases LIF, IGF-1 and uPA, and is associated with increased muscle mass, myofiber diameter, and improvement in muscle function [219]. OPN affects both immune and myogenic cells, but it is not clear which cell is responsible for its secretion, or whether its effect on muscle tissue is direct or indirect.

TREATMENT OF MUSCULAR DYSTROPHIES: WHAT ABOUT MACROPHAGES?

Excessive inflammation within dystrophic muscle is demonstrably more deleterious than beneficial. The only treatment that has shown a delay in disease progression is the use of glucocorticoids. Unfortunately, these potent anti-inflammatory drugs have significant side effects. Prednisone, one of the most widely used glucocorticoids, decreases inflammation and delays the progression of DMD, prolonging ambulation and modestly improving muscle strength and cardiopulmonary function. Side effects include bone fragility, weight gain, mood changes, and even muscle weakness [220–224]. Glucocorticoids stimulate the AKT1/FOXO1 pathway, which decreases protein synthesis and increases protein catabolism and is responsible for the seemingly contradictory muscle weakness and atrophy observed in patients treated with this drug [225]. Another potent glucocorticoid is Dexamethasone, but side effects are severe, making it an unappealing candidate for long-term treatment. Deflazacort is a less potent glucocorticoid that has a similar effect to prednisone but with a reduced number of side effects [224, 226]. In the end, a combination of the different drugs seems the most appropriate way to delay MDs progression [227]. The main concern stays the poor knowledge of long-term effects. For example, *mdx* mice treated with prednisone for 50 days showed an improvement in early disease progression, which was subsequently lost when treatment was continued to 100 or 150 days [221]. In addition, whether the effects of corticosteroids are mainly through abatement of inflammation or though one of the other pleiotropic effects of these compounds is not yet clear.

Therapeutic approaches that harness macrophages are beginning to emerge. 24 hours after an acute ischemia/reperfusion injury, intra-muscular injection of pro-inflammatory macrophages has been shown to improve muscle regeneration, characterized by increased muscle force, myofiber diameter and by decreasing collagen deposition at 14 days post-reperfusion [228]. At 5 and 7 days after reperfusion, a decrease in damaged muscle area is observed (probably via improved removal of dead cells). While the total number of macrophages was unchanged between control and the macrophage-injected muscles, an increase in the number of $CD206^+$ macrophages was observed 5 days post-perfusion in the macrophage-injected muscle, demonstrating that the injected pro-inflammatory macrophages switched toward a pro-regenerative phenotype within the treated muscle [228]. Injection of human macrophages into damaged muscle of immuno-suppressed mice, together with human myoblasts, improved myoblast proliferation and led to better host cell integration within the myofibers [229]. As observed in mice, five days after their injection pro-inflammatory human macrophages expressed anti-inflammatory markers, further demonstrating their capacity to locally change phenotype during progression of muscle regeneration [229]. Of note, one of the main causes of failure of cell therapy for MDs is the poor survival and migration capacity of SCs and myoblasts after intramuscular injection. Co-injection of BMDM and SCs into *mdx* muscle increases their proliferation, survival and migration [51]. Thus, "non-dystrophic" macrophages seem to support injected myoblasts in their regeneration of muscle tissue. Modification of macrophage phenotype could be beneficial to dystrophic muscle not only because macrophages act negatively on fibrogenic cells within the progression of MD, but also because of the evidence supporting their use in these cell therapies. Interestingly, Novak et al. demonstrated that myoblasts are not the only cells capable of delivering phosphorodiamidate morpholino oligomers to myofibers, and that

macrophages are also potent releasers of these therapeutics, making them an attractive candidate for *in situ* delivery to myoblasts and myofibers [230].

CONCLUSION

Macrophages have been studied for decades and most of their functions are now understood. Indeed, their role beyond infection response, specifically as tissue resident cells involved in the remodelling of tissues (development, regeneration and repair) are well understood within the research community. However, the manipulation of their inflammatory state in order to direct their trophic functions toward tissue-resident cells is far from being defined. MDs, and especially DMD are multifactorial diseases where necrosis, chronic inflammation, defects in angiogenesis, fibrofatty infiltration, and tissue remodelling occur asynchronously within the tissue. The defect in macrophage function in these diseases could be one of the reasons for poor outcome of cell and gene therapies. It is therefore important that further efforts be made to safely manipulate macrophage dynamics so that they might be used to therapeutic effect as part of a MD rescue approach. Today, single cell technology such as CITE-seq (Cellular Indexing of Transcriptomes and Epitopes by Sequencing) should allow the community to link macrophage function, polarization state and gene expression, to find appropriate therapeutic gene and protein targets. We imagine a future where "re-booting" or resynchronizing the inflammatory system would allow improvements to the muscle repair cycle, by delaying fibrosis apparition, and the loss of muscle function; or as synergistic tools used alongside gene and cell therapies to improve their efficacy.

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