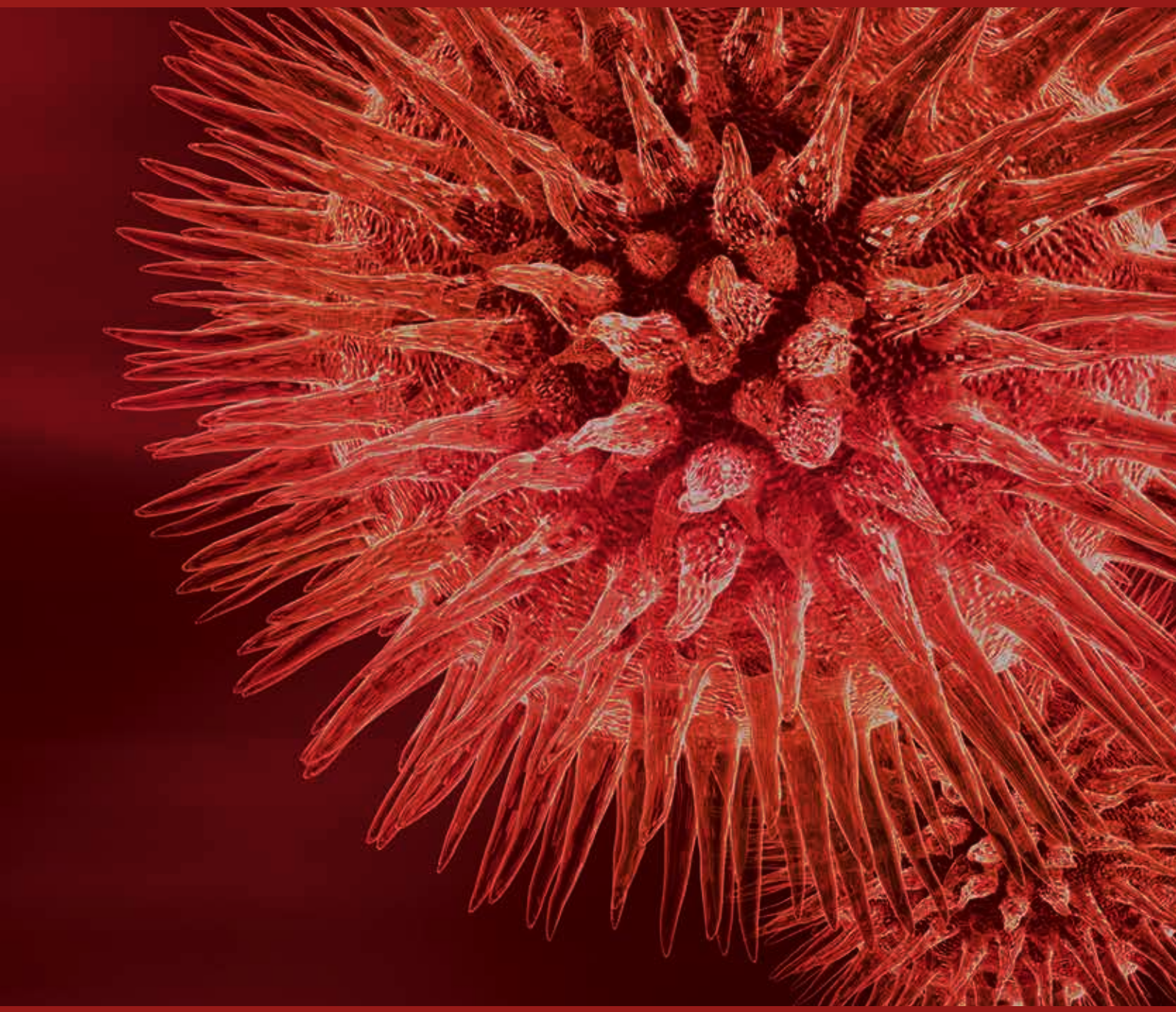
BioMed Research International

Inflammation in Muscle Repair, Aging, and Myopathies

Guest Editors: Marina Bouché, Pura Muñoz-Cánoves, Fabio Rossi, and Dario Coletti



Inflammation in Muscle Repair, Aging, and Myopathies

BioMed Research International

Inflammation in Muscle Repair, Aging, and Myopathies

Guest Editors: Marina Bouché, Pura Muñoz-Cánoves, Fabio Rossi, and Dario Coletti

Contents

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 821950, 3 pages
http://dx.doi.org/10.1155/2014/821950

Editorial

Inflammation in Muscle Repair, Aging, and Myopathies

Marina Bouché,[1] Pura Muñoz-Cánoves,[2] Fabio Rossi,[3] and Dario Coletti[4]

[1] *DAHFMO, Unit of Histology and Medical Embryology, Sapienza University of Rome, Via Antonio Scarpa 14, 00161 Rome, Italy*
[2] *Cell Biology Group, Department of Experimental and Health Sciences, Pompeu Fabra University (UPF), CIBER on Neurodegenerative Diseases (CIBERNED), Institució Catalana de Recerca i Estudis Avançats (ICREA), Doctor Aiguader 88, 08003 Barcelona, Spain*
[3] *The Biomedical Research Centre, UBC, Vancouver, BC, Canada V6T 1Z3*
[4] *B2A Biology of Adaptation and Aging, Pierre and Marie Curie University (Paris 6), 7 quai St Bernard, 75252 Paris Cedex 5, France*

Correspondence should be addressed to Marina Bouché; marina.bouche@uniroma1.it

Received 17 July 2014; Accepted 17 July 2014; Published 4 August 2014

Numerous recent studies have expanded our knowledge on the complexity of the immune system and its contribution to skeletal muscle repair, aging, and myopathies. Indeed, it is becoming clear that a precisely regulated cross talk between muscle and immune cells, involving endocrine/paracrine and cell-cell contact interactions, is required for muscle repair and maintenance of muscle homeostasis. Alterations of these mechanisms lead to unsuccessful repair in response to direct mechanical trauma (acute injury) or following secondary damage as a consequence of aging or genetic neuromuscular defects. In fact, though the capacity of muscle to regenerate relies primarily on a specific population of muscle stem cells, named satellite cells, the inflammatory cells that infiltrate the injured muscle appear to be as critical for successful regeneration. Conversely, if damage persists, as in chronic myopathies, inflammatory cell infiltration is perpetuated and leads to progressive muscle fibrosis, thus exacerbating disease severity.

While the number of scientific publications on the topic of skeletal muscle inflammation has steadily grown over the last two decades, the notion of inflammation as a common feature in muscle degeneration occurring in aging and myopathies and its association with altered muscle has to our knowledge never previously been addressed and discussed in dedicated journal issues before.

The main focus of this special issue is to bring together studies that used different experimental approaches *in vivo* or *in vitro* to dissect the dynamic changes taking place in specific immune cell populations, their cross talk with other cell types within the muscle milieu, and their contribution to normal versus pathological muscle repair.

Among the seven review articles, E. Rigamonti et al. discuss the current available literature about the role of macrophage populations in tissue injury and repair with a particular focus on skeletal muscle regeneration. Beyond their role in innate immunity, macrophages are now recognized as crucial players in orchestrating the healing of various injured tissues, including skeletal muscle. In this review the authors discuss the involvement of specific macrophage subpopulations in these processes and the complexity of defining macrophage subpopulations *in vivo*, since the *in vitro* criteria used to define M1 and M2 macrophages cannot be strictly applied to *in vivo* settings. Indeed, it is still a matter of debate whether the sequential presence of different macrophage populations results from a dynamic shift in macrophage polarization or from the recruitment of new circulating monocytes. Thus, to identify the molecular determinants of macrophage polarization is certainly one of the major tasks in developing effective targeted therapies for genetic defects of the tissue and muscle diseases associated with chronic inflammation.

However, although macrophages are emerging as indispensable for damage control and tissue remodeling following muscle injury and as principal mediators of pathological skeletal remodeling in diseases such as idiopathic inflammatory myositis (IIMs) and muscular dystrophies (MDs),

the involvement of other immune cells in promoting or preventing muscle damage resolution is also emerging. The current knowledge and recent advances on the involvement of different innate immune cells in MDs and the emerging role of additional cell populations within the acquired immune response are being discussed in the review article by L. Madaro and M. Bouché.

It is widely believed that any disruption in the coordinated initiation, progression, and resolution of inflammation can lead to persistent muscle damage and impairment of regeneration, which in many cases is also characterized by development of fibrosis, as observed in MDs. Indeed, the process of fibrosis, whose underlying mechanisms are not fully elucidated yet, represents one important deleterious consequence of impaired muscle repair. In this special issue, Y. Kharraz et al. discuss new developments in our understanding of the mechanisms leading to fibrosis in Duchenne muscular dystrophy (DMD) and several recent advances towards reverting it, as well as potential treatments to attenuate disease progression.

Although blunting inflammation would not restore the primary defect in MDs, the emerging consensus is that multiple strategies addressing different aspects of the pathology, which may eventually converge, may be successful. Indeed, various therapies under development are directed toward rescuing the dystrophic muscle damage using gene transfer or cell-therapy.

A. Farini et al. discuss current knowledge about involvement of immune system responses to experimental therapies in MDs and how the different components of the immune system response are differently activated against cell- or gene-therapy both in a specific manner, linked to the type of cell or vector used, and according to MDs-specific immune-pathogenetic mechanisms.

In this context, S. M. Maffioletti et al. give an in-depth overview of the main players and issues involved in the mechanisms and dynamics that impair the efficacy of cell transplants in DMD and discuss potential approaches that might be beneficial for future regenerative therapies of skeletal muscle.

Muscle wasting linked to immune response is not, however, a feature restricted to genetic diseases.

In cancer cachexia, for example, inflammatory cytokines are important not only to establish tumor-host interaction and deregulate inflammatory response to tumor burden, but also to mediate muscle wasting by directly targeting muscle tissue. In the review by J. K. Onesti and D. C. Guttridge a summary of the clinical implications, background of inflammatory cytokines, and the origin and sources of procachectic factors is provided. Molecular mechanisms and pathways are also described to elucidate the link between the immune response caused by the presence of the tumor and cytokine-dependent inhibition of muscle regeneration, ultimately resulting in muscle wasting.

Muscle wasting is also a feature of IIMs, a wide range of autoimmune diseases, characterized clinically by reduced muscle endurance and weakness, chronic inflammation, and infiltration by immune/inflammatory cells in skeletal muscles. Treatments for IIMs are based on lifelong immunosuppressive therapy, which comes with well-known adverse effects; recovery is incomplete for many patients. More effective therapies, with reduced side effects, are highly desirable. In this context, C. Crescioli proposes vitamin D receptor (VDR) agonists as candidates in future treatment of IIMs. In her review she summarizes the pleiotropic anti-inflammatory properties of VDRs with potentially limited adverse effects.

Similarly, A. Costa et al., in one out of the four original contributions in this issue, propose Arg-vasopressin- (AVP-) dependent pathways as an interesting strategy to counteract muscle decline in aging or myopathies. Indeed, the authors show that overexpression of the AVP receptor V1a in skeletal muscle *in vivo* increases the expression of regenerative markers, modulates immune response, and attenuates fibrogenesis, thus enhancing muscle regeneration and counteracting the negative effects of the proinflammatory cytokine TNF.

On the other hand, M. Pelosi et al. addressed the effects of IL-6, a multifaceted pleiotropic cytokine, on the myogenic program. In physiological conditions IL-6 is required for muscle homeostasis, but in several pathological conditions its level increases and it contributes to muscle wasting. In this paper the authors explored the molecular mechanisms underlying IL-6-dependent inhibition of the myogenic program and identified potential molecular mediators of these effects.

It is well known that the inflammatory response plays an important role also in the development of ischemic heart disease. In this context, G. D. Duerr et al. demonstrate in this issue that osteopontin (OPN) has a cardioprotective effect. OPN is a matricellular protein and cytokine involved in the regulation of macrophage function and as a remodeling-associated mediator in different tissues. Using *in vivo* models they show that lack of OPN prevents heart remodeling in a murine model of ischemic cardiopathy, altering the expression of contractile elements and chemokines, as well as remodeling factors. These findings may further support the therapeutical perspective for osteopontin as a possible target in protection of the ischemic heart.

To conclude this special issue, C. Sciorati et al. characterize the ability of the 7-Tesla magnetic resonance imaging (MRI) to reveal specific inflammatory events in the skeletal muscle using a mouse model of IIM. One of the limits in understanding the etiopathogenesis of muscle inflammatory diseases is the paucity of approaches for the noninvasive study of inflamed tissues. The authors provide an in-depth noninvasive characterization of this myositis model, proving the efficacy of MRI as an informative and noninvasive analytical tool for studying *in vivo* immune-mediated muscle involvement. Indeed, the availability of noninvasive tools to monitor muscle inflammation will permit reduction in the number of animals necessary for experimental studies and will increase the amount of relevant information that can be derived from single animals followed over time. At the same time, this imaging modality will complement information derived from histopathological studies that encompass only limited areas of tissue and therefore suffer from potential sampling bias.

What emerges from this special issue is that inflammation-regulated muscle repair in aging and disease is an active and rapidly advancing research field, and we hope that the papers here collected will help to connect fundamental science with biomedical research on severe myopathies, with the aim of opening new venues for therapy. It is our wish that these papers will also be a useful tool for the inflammation and skeletal muscle research community and that they will attract and motivate investigators from different scientific areas to this important field of research.

Acknowledgments

We would like to thank the BMRI Editorial Office for the opportunity of producing this special issue and all contributing authors who made this possible. Our sincerest thanks also go to the outstanding external reviewers for their expert assistance.

Marina Bouché
Pura Muñoz-Cánoves
Fabio Rossi
Dario Coletti

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 964010, 12 pages
http://dx.doi.org/10.1155/2014/964010

Review Article

Stem Cell Transplantation for Muscular Dystrophy: The Challenge of Immune Response

Sara Martina Maffioletti,[1] Maddalena Noviello,[2] Karen English,[3] and Francesco Saverio Tedesco[1]

[1] *Department of Cell and Developmental Biology, University College London, London WC1E 6DE, UK*
[2] *Experimental Hematology Unit, Division of Immunology, Transplantation and Infectious Diseases, San Raffaele Scientific Institute, 20132 Milan, Italy*
[3] *Institute of Immunology, Department of Biology, National University of Ireland Maynooth, County Kildare, Ireland*

Correspondence should be addressed to Francesco Saverio Tedesco; f.s.tedesco@ucl.ac.uk

Received 14 February 2014; Accepted 5 June 2014; Published 26 June 2014

Academic Editor: Fabio Rossi

Treating muscle disorders poses several challenges to the rapidly evolving field of regenerative medicine. Considerable progress has been made in isolating, characterizing, and expanding myogenic stem cells and, although we are now envisaging strategies to generate very large numbers of transplantable cells (e.g., by differentiating induced pluripotent stem cells), limitations directly linked to the interaction between transplanted cells and the host will continue to hamper a successful outcome. Among these limitations, host inflammatory and immune responses challenge the critical phases after cell delivery, including engraftment, migration, and differentiation. Therefore, it is key to study the mechanisms and dynamics that impair the efficacy of cell transplants in order to develop strategies that can ultimately improve the outcome of allogeneic and autologous stem cell therapies, in particular for severe disease such as muscular dystrophies. In this review we provide an overview of the main players and issues involved in this process and discuss potential approaches that might be beneficial for future regenerative therapies of skeletal muscle.

1. Introduction

Stem cell therapies hold promises for a plethora of conditions involving the loss or damage of resident tissue progenitors, including skeletal muscle. Skeletal muscle is the most abundant human tissue and its accessibility makes it a good candidate for protocols based upon the delivery of stem cells as a medicinal product. Disorders affecting skeletal muscle can be acute, such as trauma-related tissue damage or loss, and chronic, such as tissue wasting in muscular dystrophies, as typical of Duchenne muscular dystrophy (DMD), the most common paediatric inherited muscle disorder. DMD is an X-linked progressive and degenerative myopathy characterised by muscle wasting and weakness, which ultimately leads to loss of ambulation in puberty, cardiac and respiratory involvement, and premature death [1].

Different cell therapy strategies have been tested, in particular for chronic skeletal muscle disorders, using diverse types of cells with myogenic potential derived from muscle (e.g., satellite cells/myoblasts, muscle derived stem cells), vessels (e.g., pericytes and their progeny, mesoangioblasts), bone marrow, blood, or embryonic tissues, including, recently, induced pluripotent stem cells (reviewed in [2]). Some of these cells, such as mesoangioblasts, are currently completing clinical experimentation for DMD. However, the data obtained from this multitude of studies resulted in promising but suboptimal efficacy in restoring functional skeletal muscle tissue. Therefore, there is still no efficacious cell therapy-based treatment for muscle diseases. The reasons behind this are linked to challenges associated with the medicinal product (myogenic stem cells) and with the target tissue, the multinucleated, abundant, and widespread skeletal muscle [3]. General bottlenecks of cell therapies are represented by the availability of an adequate number of stem cells to transplant, which includes problems related to the harvesting from donors or from the same patient, genetic

correction (in case of autologous transplant), maintenance of myogenic potential prior to transplantation, and large scale amplification in culture under appropriate conditions and by their compatibility with the host immune system. Specific hurdles related to skeletal muscle are due to some of the tissue's intrinsic features. First of all, skeletal muscle is the most abundant tissue in the human body (several kilograms per individual) and hence cell replacement strategies require high numbers of transplantable progenitors (several million per kilogram). Moreover, the administration route greatly influences the extent of grafting [4]. Indeed transplanted cells undergo a limited, although variable, migration from the site of injection that decreases the efficiency of the treatment. Intra-arterial delivery of the cells is an alternative, but it is limited to cells that have the ability to cross the vessel wall (such as pericyte-derived mesoangioblasts and $CD133^+$ cells) [2]. This issue might be of minor relevance for the treatment of localized disorders but remains one of the most important to be overcome for the treatment of systemic muscle pathologies.

In addition to the aforementioned problems, a complex immune response further complicates and impairs the outcome of cell transplants. Data from myoblast transplantation studies indicate that 90% of donor cells are cleared within the first hour after transplantation by cell-mediated immune responses [5–7]. Moreover, muscles affected by chronic diseases are in a state of persistent inflammation and are characterized by an abundant infiltrate of immune cells that may hamper extensive grafting, proliferation, and differentiation of the transplanted stem cells into functional muscle tissue. The aim of this review is to give a general overview on the role of the immune system in the context of skeletal muscle regeneration focusing on the interaction of immune cells and transplanted stem cells in cell therapy strategies for muscular dystrophies. Inflammatory myopathies [8] represent another broad spectrum of muscle disorders with a predominant immunological aspect. Although in this type of disorder the immune system plays a primary role in provoking the muscle pathology, this will not be discussed here as it goes beyond the scope of this review.

2. Immune Response during Muscle Regeneration

Skeletal muscle originates from embryonic mesoderm and each muscle is composed of several muscle fibres (its functional unit). Each myofibre is a large syncytium containing numerous nuclei within the same cytoplasm [9]. The fibres' plasma membrane (also known as sarcolemma) is in tight contact with the satellite cells, the main resident stem cell population of skeletal muscle [10, 11]. The satellite cell niche is indeed localised between the basement membrane encircling each myofibre and the fibres' sarcolemma. Skeletal muscle has a conspicuous regenerative ability and relatively large injuries can be repaired in a few weeks. Upon activation satellite cells produce transit-amplifying progenitors called myoblasts (which will fuse with preexisting fibres or generate new fibres) and give also rise to stem cells able to maintain the pool of undifferentiated satellite cells for further rounds of regeneration (reviewed in [12]). This regeneration process is tightly orchestrated and entails the interplay of different cell types of muscle origin but also inflammatory and immune cells (Figure 1). Indeed the latter plays a very important role in all the stages of the process and alterations to any of the components impair the regenerative response.

2.1. Muscle Regeneration in Acute Injury. In skeletal muscle, acute injury either by myotoxin injection, freeze, crush, or exercise-related damage triggers a stereotypical response. Injury initiates an innate immune response characterized by proinflammatory cytokines. Soon after damage, a wave of neutrophils invades the area with a peak in their concentration at 24 hours followed by a rapid decrease [13]. Neutrophils release proinflammatory molecules (such as CXCL8 and interleukin-6 (IL-6)) that recruit macrophages into the tissue. Resident macrophages, present in the interstitial spaces of the epimysium and perimysium, play key roles especially in the first phases of acute injury [14]. Macrophages are rapidly activated and polarized towards a M1 inflammatory phenotype ("classically activated" macrophages). Neutrophils and M1 macrophages produce an array of molecules, including cytokines, chemokines, nitric oxide, and prostaglandins that sustain and amplify local inflammation [15].

Tumour necrosis factor α (TNFα) is the main proinflammatory cytokine present upon skeletal muscle damage [16]. It is initially released by degranulation of resident mast cells followed by infiltrating neutrophils and macrophages [17, 18]. Importantly, TNFα promotes activation and proliferation of satellite cells [19] while inhibiting their differentiation [20–22]. These effects are mediated by the activation of nuclear factor kappa B (NF-κB) [23].

Chemokine (C-C motif) ligands 2, 3, and 4 (CCL2, CCL3, and CCL4) are chemoattractant molecules that play a significant part in muscle regeneration and their receptors, CCR2 and CCR5, are upregulated following skeletal muscle injury [24, 25]. Data obtained from knock-out mice showed that CCR2 is required for macrophage invasion of the injury site, with impaired regeneration in CCR2-null mice in parallel with a slowed revascularization of the injured area [26]. Moreover, satellite cells and myoblasts constitutively express CCR1, CCR2, CCR4, and CCR5 [27].

M1 macrophages express the inducible nitric oxide synthase (iNOS) and hence release nitric oxide (NO) in the injury site. Although NO can damage muscle cell membranes, it also facilitates tissue debris clearance by targeting them for phagocytosis [28]. In addition, oxidative stress caused by NO stimulates satellite cell proliferation [29], a process essential for muscle regeneration.

Other key molecules present in the inflammatory phase of muscle regeneration are prostaglandins. These signalling molecules are derived from the metabolism of arachidonic acid by cyclooxygenases (COX) and expression of COX-1 and COX-2 is indeed very high in injured muscles [30]. The effects of prostaglandins have been reported in all the stages of muscle regeneration, from satellite cells proliferation [31] to differentiation [32] and fusion [33].

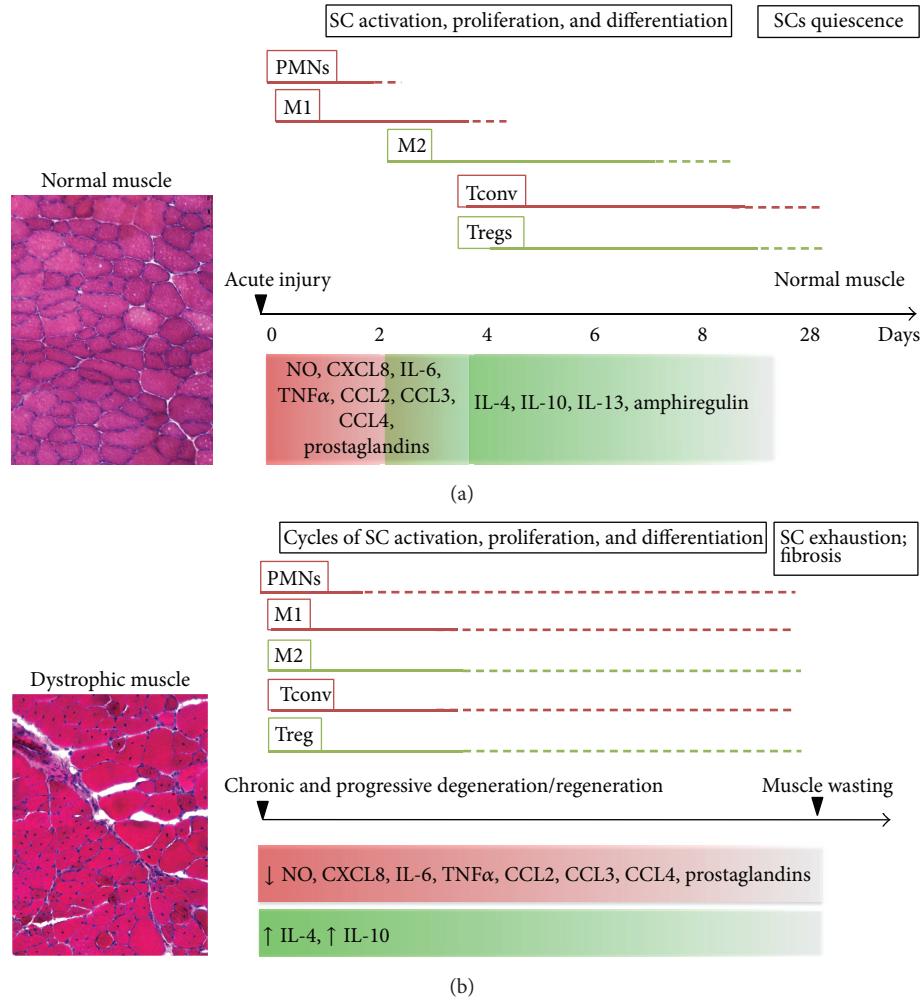


Figure 1: Dynamics of inflammation and muscle regeneration in acute and chronic injury. Acute muscle injury (a) triggers local release of chemoattractants that induce PMNs and M1 invasion into the damaged tissue. PMNs and M1 release an array of molecules (such as NO) that further amplify local inflammation, contributing to debris clearance and SC activation. This initial Th1-driven inflammation is later overcome by an anti-inflammatory response that coincides with a M1-to-M2 switch. By day 4 Tregs reach the site of injury, modulating Tconv expansion and activation and SC differentiation through amphiregulin release. M2 and Th2 cytokines reduce local inflammation and contribute to SCs differentiation, thus promoting the latest stages of muscle regeneration. Upon damage repair, SCs return to quiescence. Chronic muscle injuries (b) are characterised by persistent inflammation. Muscles feature infiltrates of PMNs, M1 together with M2, Tconv, and Treg; moreover, the simultaneous release of pro- and anti-inflammatory molecules promotes incomplete tissue regeneration and fibrosis. The SC pool undergoes depletion due to continuous rounds of activation and differentiation, resulting in terminal muscle wasting. SC: satellite cells; PMN: neutrophils; M1: M1 macrophages; M2: M2 macrophages; Tconv: $CD4^+$ conventional T cell; Tregs: $CD4^+$ $Foxp3^+$ regulatory T cell; NO: nitric oxide; Red box: Th1-driven response; Green box; Th2-driven response.

The M1-driven tissue inflammation is gradually overcome by an anti-inflammatory response due to a switch in macrophage phenotype, from M1 to M2 ("alternatively activated") [34]. M2-polarized macrophages are activated by anti-inflammatory Th2 cytokines such as IL-4, IL-10, and IL-13 that attenuate the inflammatory response through M1 deactivation [35]. Moreover, IL-4 and IL-10 act directly on muscle cells, inducing myogenin expression and subsequent differentiation and fusion [34, 36]. Hence, while M1 macrophages contribute to creating an inflammatory environment that helps clearing cell debris and activating satellite cells, M2 macrophages reduce inflammation and promote myogenic differentiation.

Although molecules are secreted by immune cells shape regeneration, the muscle is not a passive bystander. Indeed it releases a series of cytokines and chemokines collectively referred to as "myokines" [37] which also include IL-6, TNFα, and CCL2 [38, 39]. Aside from proinflammatory functions, IL-6 was demonstrated to have both an autocrine function on satellite cell proliferation and muscle hypertrophy [40] and paracrine effects when released into the circulation (on glucose metabolism and lipolysis) [41].

2.2. Regeneration in Chronic Muscle Disorders. While acute muscle injuries are characterized by a self-limiting physiological inflammatory reaction, chronic muscle conditions are generally associated with persistent inflammation. Recent data indicate that inflammation plays an active part in the pathology [42]. Chronic myopathies are a heterogeneous group of diseases characterized by progressive muscle wasting and include muscular dystrophies (e.g., DMD), which will be the focus of this review.

The important role of inflammation in muscular dystrophies is supported by the efficacy of corticosteroid treatments in improving muscle strength and function in the short term in patients [43]. Indeed muscular dystrophies are generally characterized by an infiltrate of inflammatory neutrophils and phagocytic M1 macrophages that produce inflammatory cytokines and NO, as in the case in acute injuries [44]. A hallmark of chronic muscle pathologies is the infiltration of M2 macrophages at early stages; this differs from acute muscle injury, where M2 macrophages invade the tissue only at later time points. M2 macrophages express the enzyme arginase, which shares its substrate (arginine) with iNOS expressed by M1 macrophages and this M1-M2 substrate competition decreases NO production [44]. M2 macrophages also induce a Th1-Th2 shift of cytokine production, with an increase of IL-4 and IL-10, which can deactivate M1 macrophages [35]. This Th2-dominated environment also induces the activation of eosinophils that promote muscle fibrosis through major basic protein-1-mediated processes [45]. Data obtained from mdx mice and DMD patients suggest that, besides the innate immune response, some degree of cellular immunity is also involved [46, 47]. DMD muscles are in fact characterized by the presence of infiltrating T cells expressing a highly conserved peptide in the hypervariable domain of the T-cell receptor, suggesting a breakdown in peripheral tolerance [46, 48]. Recently, dystrophin-specific T-cell responses have been described in a considerable proportion of DMD patients. Of interest, the incidence of such responses was lower in the cohort of patients receiving deflazacort (a steroid) than in untreated ones, suggesting that the modulation of cellular immunity may contribute to the beneficial effect of corticosteroid treatment [48].

Other chronic disorders characterised by an altered regenerative and inflammatory pattern in the affected muscles include, for example, the groups of facioscapulohumeral muscular dystrophy (FSHD) and the limb girdle muscular dystrophies (LGMDs). Although these disorders are caused by different genetic alterations, both FSHD and LGMD have been shown to present clear hallmarks of inflammation (e.g., in FSHD1A [49] and LGMD2B [50]). However, the relevance to the onset and progression of the pathology remains ambiguous.

3. Immunologically Relevant Molecules Expressed by Muscle Cells

In both physiological and pathological conditions, there is an active interplay between muscle cells and cells of the immune system. This interaction is made possible by a shared panel of soluble and transmembrane molecules that transduce signals and form functional synapses between the two cell types [51, 52]. Beyond the already discussed soluble factors (cytokines and chemokines) and their receptors, muscle expresses other immunologically relevant molecules.

Toll-like receptors (TLRs) are the principal activators of the innate immune response. TLRs are expressed on multiple cell types (such as dendritic cells and macrophages) and generally respond to "danger signals" (e.g., pathogens and damage associated molecules) triggering the production of inflammatory cytokines and chemokines. *In vitro* studies demonstrated that murine myotubes express TLR2, TLR4, TLR5, and TLR9 [53]. Notably, a study showed that TLR3 is expressed in muscle biopsies of patients with chronic myopathies and that TLR3 activation on human myoblasts triggers a downstream cascade leading to NF-κB activation and ultimately IL-8 production [54].

In vitro, myoblasts constitutively express major histocompatibility complex I (MHC I), which is upregulated upon inflammatory stimuli, such as IFNγ. IFNγ also induces the expression of MHC II in muscle cells [55, 56]. *In vivo*, muscle fibres do not express MHC I or MHC II under physiological conditions [57], but they are expressed at high levels in inflammatory muscle disorders [58]. Due to the inducible MHC I/II expression, muscle cells are considered to be nonprofessional antigen presenting cells (APCs) and thus have the capacity to trigger T-cell-mediated immune response. In this context, it was demonstrated that human muscle cells possess all the intracellular machinery required for antigen processing in the context of MHC I/II presentation [59, 60]. Moreover, presence of fibroblast growth factor (FGF) in dystrophic muscle [61] may on the one hand regulate proliferation of myogenic progenitors [62] but on the other hand lead to expression of the MHC class II receptor HLA-DR, as it has been identified in human mesenchymal stem cells [63]. Aside from MHC molecules, myoblasts can express a nonclassical MHC I molecule, human leukocyte antigen-G (HLA-G), under inflammatory conditions [64]. HLA-G is a molecule with very low polymorphism and it is generally considered tolerogenic because of its role in maternal-foetal tolerance [65] and graft acceptance [66].

Canonical costimulatory molecules, such as the CD80 and CD86, required together with MHC for T-cells activation, have not been identified on muscle cells [67, 68]. CD40-CD40 ligand interaction is another key signal required in both humoral and cellular immunity. CD40 is usually present on the membrane of APCs and CD40 ligand on activated $CD4^+/CD8^+$ T cells, where it transduces signals for their stimulation and expansion. CD40 is also found expressed in other cell types [69, 70], including muscle cells. *In vitro*, human myoblasts constitutively express CD40 and its levels increase upon IFNγ and TNFα stimulation [67]. In this cell type, CD40-CD40 ligand interaction leads, among other effects, to an increase of intracellular adhesion molecule 1 (ICAM-1) expression [71] and hence to interaction with T cells present in the muscle tissue.

Adhesion molecules have a pivotal function in allowing interaction of muscle cells with immune cells. During skeletal muscle inflammation, ICAM-1 is expressed by both

the endothelium of the vessels and by the muscle fibres [51, 72, 73]. ICAM-1 interacts with leukocyte function-associated antigen 1 (LFA-1), an integrin, which is expressed on T cells. ICAM-1/LFA-1 interaction leads to T-cell recruitment into the inflamed tissue (via ICAM-1 expression on endothelial cells) facilitating myofibre cytotoxicity (through myofiber-$CD8^+$ T-cell interaction) [74].

In addition, PD-1/PD-L1 binding is thought to play an important role in suppressing immune responses [75]. Interestingly, expression of programmed death ligand 1 (PD-L1), an immune-inhibitory molecule, is induced in mesoangioblasts stimulated with proinflammatory cytokines [76] and in muscle biopsies from patients with idiopathic inflammatory myopathies [77]. Based on these findings, these cells are more likely to perform crucial functions in limiting, rather than priming, a muscle-directed immune response in inflammatory settings.

4. Interaction between Immune Cells and Muscle Stem Cells: *In Vitro* Studies

A large body of data provides evidence for the important part played by immune cells in shaping the regenerative response following muscle damage/injury. We have already discussed the key cells involved in this process and their role in promoting repair; however, much less is understood about the interactions between immune cells and muscle stem cells. Studies have shown the capacity for macrophages and macrophage-conditioned medium to enhance myogenic precursor cell adhesion and migration *in vitro* [78–80]. Blocking studies revealed pivotal functions for TNF-α and high mobility group box 1 (HMGB1) protein likely derived from macrophages [79, 80].

Dendritic cells (DC, professional APCs) have been identified in inflammatory infiltrates in muscle biopsies and may play an essential role in direct activation of antigen specific T cells [81]. Coculture of DC with myoblasts leads to a semimature DC phenotype with reduced ability to promote T-cell activation and proliferation in a cell contact dependent manner [82]. This study provided one of the first observations that muscle progenitor cells have immune modulatory capacity and could potentially promote a tolerogenic environment.

The adaptive immune response also has a role in inflammatory muscle disorders. Following activation by the innate immune system, dystrophin reactive T cells have been identified in DMD patients [48]. As such, these T cells may pose a threat both to autologous gene-corrected cell therapies and also to allogeneic cellular therapies. One *in vitro* study has investigated the effects of T cells on human mesoangioblasts and vice versa [76]. Despite an increase in HLA molecules expressed by mesoangioblasts following stimulation with IFN-γ, these cells failed to induce T-cell proliferation *in vitro*. Mesoangioblasts expressed low or negligible levels of the costimulatory molecules CD40, CD80, and CD86; however, following stimulation with proinflammatory cytokines significant expression of the inhibitory molecule PD-L1 was observed. This suggested that mesoangioblasts are hypoimmunogenic [76]. This study also examined the effects of mesoangioblasts on T cells and their immunosuppressive capacity *in vitro*. Mesoangioblasts suppressed both $CD4^+$ and $CD8^+$ T-cell proliferation in a dose and time dependent manner but did not induce anergy in T cells. In addition, mesoangioblasts inhibited T-cell production of proinflammatory cytokines. The mechanisms of action identified an important role for IFN-γ and TNF-α in activation of mesoangioblast to become immunosuppressive followed by secretion of indoleamine 2,3-dioxygenase (IDO) and prostaglandin E2 (PGE-2) to inhibit T-cell proliferation [76]. Similar findings were obtained using induced pluripotent stem (iPS) cell-derived mesoangioblast-like cells [83]. In addition, mesoangioblasts show peculiar resistance to T-cell killing, although they are recognized and killed by allogeneic T cells in an inflammatory microenvironment or upon differentiation into myotubes [84].

Aside from the positive effects that muscle stem cells may have in promoting a tolerogenic environment, there are negative interactions between these different cell populations. As discussed previously, neutrophils and macrophages are required for clearance of cell debris among other functions. In addition, cytotoxic killing of muscle cells by neutrophils was also reported [85]. This study identified interactions between neutrophils and macrophages, which promote macrophage killing of muscle cells *in vitro*. Similarly, TLR3 stimulation of muscle cells *in vitro* leads to the upregulation of the activating receptor NKG2D and subsequent natural killer (NK) mediated lysis of muscle cells [54]. However, it may be possible to reduce the susceptibility of muscle stem cells to NK-mediated lysis. Indeed a recent study demonstrated that prestimulation with IFN-γ can significantly decrease the susceptibility of allogenic human mesenchymal stem cells to activated NK-mediated cytotoxicity *in vitro* [86]. Few studies have been carried out to investigate the interactions between these two cell populations highlighting the need for additional investigation in this area. Importantly, *in vivo* models of muscle degeneration may provide a better understanding of the interactions between muscle stem cells and immune cells and how best to facilitate successful engraftment and function of stem cells.

5. Studying Immune Response upon Muscle Stem Cell Transplantation in Preclinical Models

5.1. Animal Models of Muscular Dystrophies. Animal models are extremely useful to investigate the pathogenesis of muscular dystrophies, the contribution of inflammation and immune responses in muscle repair and to evaluate safety and efficacy of novel therapeutic strategies. In particular, several animal models were developed for muscular dystrophies, among which the most commonly used is the X-linked muscular dystrophy mouse (mdx) carrying nonsense mutation in exon 23 of the dystrophin gene [87]. For the preclinical validation of transplanted human myogenic progenitors, immunodeficient murine models have been particularly helpful to minimize xenoreactivity and to facilitate engraftment of human cells. Several immunodeficient mice modelling DMD

are available, including the nude/mdx mice [88], lacking the T-cell compartment, the SCID-mdx mice [89], lacking both T and B cells, and the recently described NSG-mdx^{4CV} [90], and Rag2^{-}IL2rb^{-}DMD^{-} mice [91], in which the NK cell activity is also defective. Dystrophic and immunodeficient mice are also available for limb-girdle muscular dystrophies (LGMD), including the alpha-sarcoglycan-null/scid/beige mouse for LGMD2D (alpha-sarcoglycan deficiency) [92] and the SCID/BlAJ mouse for LGMD2B (dysferlin deficiency) [93]. The above mouse models have been used to transplant nonsyngeneic cells harbouring additional transgenes (e.g., GFP), and some of them were also shown to be good recipients for novel human pluripotent stem cell-based protocols for muscular dystrophies [92, 94].

Among large animal models, the Golden Retriever muscular dystrophy (GRMD) [95, 96] and the Beagle-based canine X-linked muscular dystrophy (CXMD [97]) models were used in preclinical studies to demonstrate safety and efficacy of stem cell-based approaches for muscular dystrophies [98–100]. These independent studies shared the systemic delivery of nonmyoblast myogenic stem cells of mesodermal/mesenchymal origin, that is, mesoangioblasts [98], mesenchymal stromal cells (MyoD-transduced) [99] and muscle stem cells [100]. Allogeneic (with immunosuppressive therapy) and autologous gene-corrected stem cells were tested and overall results showed low frequency of inflammatory infiltrates and absence of anti-dystrophin antibodies.

Very recently the generation and characterisation of dystrophin-deficient pigs have been reported [101]. Even if this model appears to be particularly severe in comparison with the human disease progression, it could offer an additional platform for future studies. Although there are no dystrophic nonhuman primates, the use of wild type strains to optimize the design of cell therapy approaches has been reported. Several immunosuppressive regimen and cell injection modalities were compared in nonhuman primates to improve myoblast transplantation [102].

5.2. Stem Cell Transplantation. During the last decade, limitations in myoblast transplantation (detailed in the next section) fostered the search for other transplantable myogenic progenitors [2]. Given the pathological role of inflammation and immune dysregulation in muscular dystrophies [103, 104] and the high risk of rejection documented after myoblasts transplantation, many groups tried to find a transplantable cell type which combines myogenic potential together with anti-inflammatory and immunomodulatory properties. To this end, bone marrow-derived mesenchymal stromal cells (MSC, reviewed in [105]) were utilised for muscle regeneration [88]. However, a subsequent study showed that MSC engraftment into dystrophin-deficient mice did not result in spontaneous differentiation into muscle fibres and in functional recovery [106].

Mesoangioblasts are pericyte-derived progenitors that can be isolated from adult muscles and are able to differentiate into muscle fibres *in vitro* and *in vivo* upon transplantation (reviewed in [2]). The finding that allogeneic transplantation of mouse mesoangioblasts into alpha-sarcoglycan null dystrophic mice gave rise to alpha-sarcoglycan positive muscle fibres suggested that these cells may have some degree of immune evasion [107]. The immunosuppressive properties of human mesoangioblasts have been described above [76] and recent *in vitro* observations indicated that their immune privileged phenotype can be partially reverted during inflammation or upon differentiation [84]. A similar mechanism might be responsible for the negative outcome observed in a study where the alloreactive response of MSCs led to donor graft rejection [108]. Thus, although immune privileged stem/progenitors have regenerative capacity useful to treat muscle disorders, the survival of allogeneic stem cell progeny *in vivo* may still require pharmacological immunomodulation.

In the autologous setting (e.g., gene therapy strategies), host cells are genetically manipulated to correct or replace the defective gene. Based on the promising results obtained in dystrophic animals [109], clinical trials based on AAV-mediated gene transfer in muscles were designed to treat patients affected by inherited muscle disorders. However, the development of cellular and humoral responses specific for vector components [110] and/or for the transgene [111] posed important limitations and triggered further research to solve this issue and develop new gene therapy vectors [2].

One possible solution is the use of regulatory T cells (Treg) [112]. For instance, expansion of antigen specific Tregs after vector-mediated gene transfer to the liver leads to the induction of tolerance to the transgene [113, 114]. Recently, it was demonstrated that a specific subtype of clonally expanded Treg cells (specifically Foxp3^{+} CD4^{+} with a restricted TCR repertoire) was enriched in muscle upon acute or chronic injury, facilitating a nonimmunological role that favours tissue repair [115]. The authors proposed that Treg cells act, at least in part, by regulating the infiltrating myeloid population and by stimulating satellite cell proliferation and differentiation via the secretion of the growth factor amphiregulin [115].

5.3. Limitations of Xenografts. As the field of regenerative gene and cell therapy progresses and transgene expression reaches the threshold required for clinical benefit, the immune response elicited in human muscle remains a challenging issue that needs to be addressed to enhance the efficacy of these promising therapeutic approaches. Unfortunately, immunodeficient mice still show limited engraftment and are not able to predict host immune responses. Thus, further studies are needed to clarify the mechanism underlying these reactions and to identify potential targets of immune intervention. Possible options might be transplantation in juvenile mice (where the muscle is less "primed" by inflammation) [92], neonatal desensitization [116], or evasion of macrophage killing [117, 118].

Although transplantation of different human stem cell populations in immunodeficient mice allows studying their safety and efficacy profile, this assay gives only suboptimal results, possibly because of variables other than the immune system regulating donor cell engraftment. Indeed,

several species-specific mechanisms of survival, migration, and expansion and differentiation depend on the direct interaction with the host environment (e.g., integrins and other proteins of the extracellular matrix), which in the case of a xenotransplant will be significantly mismatched. Overall, experiments in small and large animals paved the way to the clinical translation of therapeutic strategies based upon the infusion of healthy donor myogenic cells. These and other studies are analysed in the next section.

6. From Preclinical Studies to Clinical Trials

Following the promising results observed in mdx mice [119], a number of clinical trials in the early 1990s tested allogeneic transplantation of myoblasts to treat muscular dystrophies (reviewed in [7, 12]). Unfortunately, the outcome was disappointing due to the limited or absent dystrophin expression and to the limited gain in muscle strength of treated patients [120]. The major limitations to the success of allogeneic myoblasts transplantation were the high early mortality rate and the limited migratory abilities of myoblasts upon transplantation, together with the host immune reaction. The group of Tremblay treated dystrophic patients with intramuscular injection of allogeneic myoblasts in the absence of immune suppression and documented acute rejection of the cells [121]. This group then reported both cellular and humoral alloreactive responses in rodents, with myoblast-injected muscles infiltrated by activated $CD4^+$ and $CD8^+$ T lymphocytes and myoblast-reactive antibodies detected in recipient sera [122]. Thus, specific immune responses against injected cells were demonstrated and claimed to explain, at least in part, the suboptimal therapeutic benefits, suggesting the need for immune suppression to avoid acute rejection. Several immunosuppressive agents were therefore tested for their ability to promote myoblast engraftment in preclinical models. Tacrolimus administration was found to adequately control immune reactions without affecting myoblast proliferation and differentiation capacity, both in mice and in nonhuman primates [102, 123].

Another bottleneck for myoblast engraftment in mice was the high mortality rate of the injected cells during the first three days after transplantation. The early loss of donor cells was explained on the one hand with the variable viability of the cell preparation and on the other with inflammation-mediated events. Neutrophils and macrophages infiltrate myoblast-injected muscles within a few hours and likely mediate early cell death before the development of adaptive immune responses [124]. Interestingly, a study on myoblast dynamics indicated that only a minority of injected cells showing stem-cell-like behaviour have the chance to survive long-term and exert regenerative capacity, suggesting that immune rejection is not the only limitation of myoblast-based therapy [125]. Furthermore, other studies excluded a role for innate immune-mediated rejection [126]. Additionally, ischaemic necrosis of implanted cells was also found to be an important hurdle to a successful graft [127]. The limited migratory ability of myoblasts required multiple injections in separated sites in nonhuman primates [102] and represents a further limitation for the treatment of muscular dystrophies affecting the majority of skeletal muscles, including the diaphragm.

Although high-density injections of allogeneic myoblasts under tacrolimus administration led to the development of new muscle fibres in DMD patients [128], the current consensus is that myoblasts transplantation can be the elective treatment only for localized forms of muscles diseases. Indeed a recent phase I/IIa clinical study trial reported some benefit using autologous myoblast transplantation in the cricopharyngeal muscles of 12 adult patients affected by oculopharyngeal muscular dystrophy (OPMD), an autosomal dominant genetic disease characterized by ptosis and dysphagia. Safety and tolerability were observed in all the patients, together with an improvement in the quality of life score. No functional degradation in swallowing was observed for 10/12 patients [129]. At variance with most autologous transplantation strategies, the above study did not require any genetic correction of the medicinal product, since it was possible to do a biopsy in several healthy muscles of the same patients. However, this would not be possible for most muscular dystrophies and a possible preexisting immunity against the vector, the mutated protein, or the newly introduced wild type epitopes needs to be taken into account, although this might not correlate directly with a negative outcome [48, 129–131].

The need to overcome the hurdles observed in myoblasts transplantation studies for DMD prompted several laboratories to identify alternative populations of myogenic cells with a better profile in terms of expansion, survival, and migration. Among these, $CD133^+$ cells and mesoangioblasts have been tested clinically. The safety of autologous transplantation of muscle-derived $CD133^+$ cells was tested in eight boys with DMD in a double-blinded phase I clinical trial and no adverse events were reported [132]. Future follow-up studies based upon genetically corrected $CD133^+$ are expected. A first-in-man phase I/II clinical trial based upon intra-arterial infusion of donor HLA-matched mesoangioblasts in 5 DMD boys receiving tacrolimus as immunosuppressive therapy is currently approaching conclusion at San Raffaele Hospital (Milan, Italy; EudraCT number 2011-000176-33). Clinical, biochemical and functional progress of the disease were followed during the year preceding treatment and were validated with a cohort of ambulant DMD boys and healthy controls [133]. Safety is the primary objective of the study and preliminary results indicate that the treatment is relatively safe. Indeed no adverse events due to immune suppression were observed, with good control of the immune response in the patients (Cossu et al., unpublished results). This study also provides clinical proof-of-principle for transplantation and intravascular delivery of nonhaematopoietic cells in DMD. Improvement of mesoangioblast extravasation upon modulation of endothelial junctional proteins in dystrophic mice has been recently published [134] and additional strategies to translate mesoangioblast transplantation to autologous settings, based upon human artificial chromosomes [135], reversible cell immortalization (Benedetti et al., in preparation) and differentiation of iPS cells [92, 136] are currently under development.

7. Conclusions

Stem cell transplantation for muscle disorders has faced several hurdles since the first trials more than 20 years ago. Progress has been made and myogenic stem cells other than myoblasts have entered clinical experimentation. Nevertheless, understanding what are the key factors allowing stable cell engraftment still remains critical for the success of allogeneic or autologous transplants in inflamed muscles. Clear immunological characterisation of stem cells (particularly when derived from pluripotent stem cells) together with a deeper understanding of the relevance of preexisting reactive T cells are issues that are undergoing intense investigation. Moreover, it is necessary to take into account other complex matters such as the insurgence of immune responses against the restored protein (e.g., against dystrophin in DMD) or against viral elements (in gene therapy settings) that might appear at different times. All these points add an additional level of complexity to the analysis of immune responses in stem cell therapies for muscular dystrophies and might require interventions beyond immunosuppression, such as induction of immune tolerance. Models and strategies to improve the outcome of xenotransplantation in immunodeficient animals will also be required in order to develop assays powerful enough to assess safety and efficacy of different types of myogenic stem cells. Even in such a case, some of the information necessary for the refinement of complex therapies (such as those based upon stem cells) will inevitably be unpredictable. However, prompt bedside-to-bench studies will bring invaluable insights to the field and, hopefully, efficacious solutions.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors would like to thank Giulio Cossu, Sara Benedetti, and Paolo Polzella for the critical reading of the paper and Tamara Casteels for the images in the figure. Work in authors' laboratories is supported by the UK Medical Research Council (Grant nos. MR/J006785/1 and MR/L002752/1), European Research Council, European Union FP7 projects Optistem, Biodesign and PluriMes (Grant Agreement no. 602423), Takeda New Frontier Science, and Duchenne Parent Project Italy.

References

[1] E. Mercuri and F. Muntoni, "Muscular dystrophies," *The Lancet*, vol. 381, no. 9869, pp. 845–860, 2013.

[2] S. Benedetti, H. Hoshiya, and F. S. Tedesco, "Repair or replace? Exploiting novel gene and cell therapy strategies for muscular dystrophies," *The FEBS Journal*, vol. 280, no. 17, pp. 4263–4280, 2013.

[3] F. S. Tedesco and G. Cossu, "Stem cell therapies for muscle disorders," *Current Opinion in Neurology*, vol. 25, no. 5, pp. 597–603, 2012.

[4] Y. Fan, M. Maley, M. Beilharz, and M. Grounds, "Rapid death of injected myoblasts in myoblast transfer therapy," *Muscle & Nerve*, vol. 19, pp. 853–860, 1996.

[5] J. R. Beauchamp, C. N. Pagel, and T. A. Partridge, "A dual-marker system for quantitative studies of myoblast transplantation in the mouse," *Transplantation*, vol. 63, no. 12, pp. 1794–1797, 1997.

[6] S. I. Hodgetts, M. W. Beilharz, A. A. Scalzo, and M. D. Grounds, "Why do cultured transplanted myoblasts die in vivo? DNA quantification shows enhanced survival of donor male myoblasts in host mice depleted of CD4+ and CD8+ cells or NK1.1+ cells," *Cell Transplantation*, vol. 9, no. 4, pp. 489–502, 2000.

[7] D. Skuk and J. P. Tremblay, "Myoblast Transplantation in Skeletal Muscles," in *Handbook of Stem Cells*, pp. 653–664, 2nd edition, 2013.

[8] J. W. Teener, "Inflammatory and toxic myopathy," *Seminars in Neurology*, vol. 32, no. 5, pp. 491–499, 2012.

[9] B. Gayraud-Morel, F. Chrétien, and S. Tajbakhsh, "Skeletal muscle as a paradigm for regenerative biology and medicine," *Regenerative Medicine*, vol. 4, no. 2, pp. 293–319, 2009.

[10] A. Mauro, "Satellite cell of skeletal muscle fibers," *The Journal of Biophysical and Biochemical Cytology*, vol. 9, pp. 493–495, 1961.

[11] F. Relaix and P. S. Zammit, "Satellite cells are essential for skeletal muscle regeneration: the cell on the edge returns centre stage," *Development*, vol. 139, no. 16, pp. 2845–2856, 2012.

[12] F. S. Tedesco, A. Dellavalle, J. Diaz-Manera, G. Messina, and G. Cossu, "Repairing skeletal muscle: regenerative potential of skeletal muscle stem cells," *Journal of Clinical Investigation*, vol. 120, no. 1, pp. 11–19, 2010.

[13] B. A. St. Pierre and J. G. Tidball, "Differential response of macrophage subpopulations to soleus muscle reloading after rat hindlimb suspension," *Journal of Applied Physiology*, vol. 77, no. 1, pp. 290–297, 1994.

[14] M. Brigitte, C. Schilte, A. Plonquet et al., "Muscle resident macrophages control the immune cell reaction in a mouse model of notexin-induced myoinjury," *Arthritis and Rheumatism*, vol. 62, no. 1, pp. 268–279, 2010.

[15] J. G. Tidball and S. A. Villalta, "Regulatory interactions between muscle and the immune system during muscle regeneration," *American Journal of Physiology*, vol. 298, no. 5, pp. R1173–R1187, 2010.

[16] G. L. Warren, T. Hulderman, N. Jensen et al., "Physiological role of tumor necrosis factor alpha in traumatic muscle injury," *The FASEB Journal*, vol. 16, no. 12, pp. 1630–1632, 2002.

[17] R. A. Collins and M. D. Grounds, "The role of tumor necrosis factor-alpha (TNF-α) in skeletal muscle regeneration: studies in TNF-$\alpha^{(-/-)}$ and TNF-$\alpha^{(-/-)}$/LT-$\alpha^{(-/-)}$ mice," *Journal of Histochemistry and Cytochemistry*, vol. 49, no. 8, pp. 989–1001, 2001.

[18] H. G. Radley and M. D. Grounds, "Cromolyn administration (to block mast cell degranulation) reduces necrosis of dystrophic muscle in mdx mice," *Neurobiology of Disease*, vol. 23, no. 2, pp. 387–397, 2006.

[19] Y.-P. Li, "TNF-α is a mitogen in skeletal muscle," *The American Journal of Physiology—Cell Physiology*, vol. 285, no. 2, pp. C370–C376, 2003.

[20] R. C. J. Langen, J. L. J. Van Der Velden, A. M. W. J. Schols, M. C. J. M. Kelders, E. F. M. Wouters, and Y. M. W. Janssen-Heininger,

"Tumor necrosis factor-alpha inhibits myogenic differentiation through MyoD protein destabilization," *FASEB Journal*, vol. 18, no. 2, pp. 227–237, 2004.

[21] D. C. Guttridge, C. Albanese, J. Y. Reuther, R. G. Pestell, and A. S. Baldwin Jr., "NF-κB controls cell growth and differentiation through transcriptional regulation of cyclin D1," *Molecular and Cellular Biology*, vol. 19, no. 8, pp. 5785–5799, 1999.

[22] R. C. J. Langen, A. M. W. J. Schols, M. C. J. M. Kelders, E. F. M. Wouters, and Y. M. W. Janssen-Heininger, "Inflammatory cytokines inhibit myogenic differentiation through activation of nuclear factor-κB," *The FASEB Journal*, vol. 15, no. 7, pp. 1169–1180, 2001.

[23] N. Bakkar, J. Wang, K. J. Ladner et al., "IKK/NF-κB regulates skeletal myogenesis via a signaling switch to inhibit differentiation and promote mitochondrial biogenesis," *The Journal of Cell Biology*, vol. 180, no. 4, pp. 787–802, 2008.

[24] M. Summan, M. McKinstry, G. L. Warren et al., "Inflammatory mediators and skeletal muscle injury: a DNA microarray analysis," *Journal of Interferon and Cytokine Research*, vol. 23, no. 5, pp. 237–245, 2003.

[25] G. L. Warren, L. O'Farrell, M. Summan et al., "Role of CC chemokines in skeletal muscle functional restoration after injury," *The American Journal of Physiology—Cell Physiology*, vol. 286, no. 5, pp. C1031–C1036, 2004.

[26] O. Ochoa, D. Sun, S. M. Reyes-Reyna et al., "Delayed angiogenesis and VEGF production in CCR2-/- mice during impaired skeletal muscle regeneration," *American Journal of Physiology*, vol. 293, no. 2, pp. R651–R661, 2007.

[27] L. Yahiaoui, D. Gvozdic, G. Danialou, M. Mack, and B. J. Petrof, "CC family chemokines directly regulate myoblast responses to skeletal muscle injury," *The Journal of Physiology*, vol. 586, no. 16, pp. 3991–4004, 2008.

[28] L. I. Filippin, M. J. Cuevas, E. Lima, N. P. Marroni, J. Gonzalez-Gallego, and R. M. Xavier, "Nitric oxide regulates the repair of injured skeletal muscle," *Nitric Oxide: Biology and Chemistry*, vol. 24, no. 1, pp. 43–49, 2011.

[29] J. E. Anderson, "A role for nitric oxide in muscle repair: nitric oxide-mediated activation of muscle satellite cells," *Molecular Biology of the Cell*, vol. 11, no. 5, pp. 1859–1874, 2000.

[30] B. A. Bondesen, S. T. Mills, K. M. Kegley, and G. K. Pavlath, "The COX-2 pathway is essential during early stages of skeletal muscle regeneration," *The American Journal of Physiology—Cell Physiology*, vol. 287, no. 2, pp. C475–C483, 2004.

[31] R. J. Zalin, "The role of hormones and prostanoids in the *in vitro* proliferation and differentiation of human myoblasts," *Experimental Cell Research*, vol. 172, no. 2, pp. 265–281, 1987.

[32] U. B. Schutzle, M. J. O. Wakelam, and D. Pette, "Prostaglandins and cyclic AMP stimulate creatine kinase synthesis but not fusion in cultured embryonic chick muscle cells," *Biochimica et Biophysica Acta*, vol. 805, no. 2, pp. 204–210, 1984.

[33] R. J. Zalin, "Prostaglandins and myoblast fusion," *Developmental Biology*, vol. 59, no. 2, pp. 241–248, 1977.

[34] L. Arnold, A. Henry, F. Poron et al., "Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis," *The Journal of Experimental Medicine*, vol. 204, no. 5, pp. 1057–1069, 2007.

[35] S. Gordon, "Alternative activation of macrophages," *Nature Reviews Immunology*, vol. 3, no. 1, pp. 23–35, 2003.

[36] V. Horsley, K. M. Jansen, S. T. Mills, and G. K. Pavlath, "IL-4 acts as a myoblast recruitment factor during mammalian muscle growth," *Cell*, vol. 113, no. 4, pp. 483–494, 2003.

[37] B. K. Pedersen, "Muscle as a secretory organ," *Comprehensive Physiology*, vol. 3, pp. 1337–1362, 2013.

[38] P. Plomgaard, M. Penkowa, and B. K. Pedersen, "Fiber type specific expression of TNF-alpha, IL-6 and IL-18 in human skeletal muscles," *Exercise Immunology Review*, vol. 11, pp. 53–63, 2005.

[39] H. Lu, D. Huang, R. M. Ransohoff, and L. Zhou, "Acute skeletal muscle injury: CCL2 expression by both monocytes and injured muscle is required for repair," *FASEB Journal*, vol. 25, no. 10, pp. 3344–3355, 2011.

[40] A. L. Serrano, B. Baeza-Raja, E. Perdiguero, M. Jardí, and P. Muñoz-Cánoves, "Interleukin-6 is an essential regulator of satellite cell-mediated skeletal muscle hypertrophy," *Cell Metabolism*, vol. 7, no. 1, pp. 33–44, 2008.

[41] B. K. Pedersen, A. Steensberg, C. Fischer, C. Keller, K. Ostrowski, and P. Schjerling, "Exercise and cytokines with particular focus on muscle-derived IL-6," *Exercise Immunology Review*, vol. 7, pp. 18–31, 2001.

[42] B. de Paepe and J. L. de Bleecker, "Cytokines and chemokines as regulators of skeletal muscle inflammation: presenting the case of duchenne muscular dystrophy," *Mediators of Inflammation*, vol. 2013, Article ID 540370, 10 pages, 2013.

[43] A. Y. Manzur, T. Kuntzer, M. Pike, and A. Swan, "Glucocorticoid corticosteroids for Duchenne muscular dystrophy," *The Cochrane Database of Systematic Reviews*, no. 1, Article ID CD003725, 2008.

[44] S. A. Villalta, H. X. Nguyen, B. Deng, T. Gotoh, and J. G. Tidbal, "Shifts in macrophage phenotypes and macrophage competition for arginine metabolism affect the severity of muscle pathology in muscular dystrophy," *Human Molecular Genetics*, vol. 18, no. 3, pp. 482–496, 2009.

[45] M. Wehling-henricks, S. Sokolow, J. J. Lee, K. H. Myung, S. A. Villalta, and J. G. Tidball, "Major basic protein-1 promotes fibrosis of dystrophic muscle and attenuates the cellular immune response in muscular dystrophy," *Human Molecular Genetics*, vol. 17, no. 15, pp. 2280–2292, 2008.

[46] E. Gussoni, G. K. Pavlath, R. G. Miller et al., "Specific T cell receptor gene rearrangements at the site of muscle degeneration in Duchenne muscular dystrophy," *Journal of Immunology*, vol. 153, no. 10, pp. 4798–4805, 1994.

[47] M. J. Spencer, E. Montecino-Rodriguez, K. Dorshkind, and J. G. Tidball, "Helper ($CD4^+$) and cytotoxic ($CD8^+$) T cells promote the pathology of dystrophin-deficient muscle," *Clinical Immunology*, vol. 98, no. 2, pp. 235–243, 2001.

[48] K. M. Flanigan, K. Campbell, L. Viollet et al., "Anti-dystrophin T cell responses in Duchenne muscular dystrophy: prevalence and a glucocorticoid treatment effect," *Human Gene Therapy*, vol. 24, no. 9, pp. 797–806, 2013.

[49] S. Hauerslev, M. C. Orngreen, J. M. Hertz, J. Vissing, and T. O. Krag, "Muscle regeneration and inflammation in patients with facioscapulohumeral muscular dystrophy," *Acta Neurologica Scandinavica*, vol. 128, no. 3, pp. 194–201, 2013.

[50] K. Nagaraju, R. Rawat, E. Veszelovszky et al., "Dysferlin deficiency enhances monocyte phagocytosis: a model for the inflammatory onset of limb-girdle muscular dystrophy 2B," *The American Journal of Pathology*, vol. 172, no. 3, pp. 774–785, 2008.

[51] D. Figarella-Branger, M. Civatte, C. Bartoli, and J. Pellissier, "Cytokines, chemokines, and cell adhesion molecules in inflammatory myopathies," *Muscle & Nerve*, vol. 28, no. 6, pp. 659–682, 2003.

[52] K. Nagaraju, N. Raben, G. Merritt, L. Loeffler, K. Kirk, and P. Plotz, "A variety of cytokines and immunologically relevant

surface molecules are expressed by normal human skeletal muscle cells under proinflammatory stimuli," *Clinical and Experimental Immunology*, vol. 113, no. 3, pp. 407–414, 1998.

[53] J. H. Boyd, M. Divangahi, L. Yahiaoui, D. Gvozdic, S. Qureshi, and B. J. Petrof, "Toll-like receptors differentially regulate CC and CXC chemokines in skeletal muscle via NF-κB and calcineurin," *Infection and Immunity*, vol. 74, no. 12, pp. 6829–6838, 2006.

[54] B. Schreiner, J. Voss, J. Wischhusen et al., "Expression of toll-like receptors by human muscle cells in vitro and in vivo: TLR3 is highly expressed in inflammatory and HIV myopathies, mediates IL-8 release and up-regulation of NKG2D-ligands," *FASEB Journal*, vol. 20, no. 1, pp. 118–120, 2006.

[55] R. Roy, G. Dansereau, J. P. Tremblay et al., "Expression of major histocompatibility complex antigens on human myoblasts," *Transplantation Proceedings*, vol. 23, no. 1, pp. 799–801, 1991.

[56] R. Mantegazza, S. M. Hughes, D. Mitchell, M. Travis, H. M. Blau, and L. Steinman, "Modulation of MHC class II antigen expression in human myoblasts after treatment with IFN-γ," *Neurology*, vol. 41, no. 7, pp. 1128–1132, 1991.

[57] G. Karpati, Y. Pouliot, and S. Carpenter, "Expression of immunoreactive major histocompatibility complex products in human skeletal muscles," *Annals of Neurology*, vol. 23, no. 1, pp. 64–72, 1988.

[58] P. Englund, E. Lindroos, I. Nennesmo, L. Klareskog, and I. E. Lundberg, "Skeletal muscle fibers express major histocompatibility complex class II antigens independently of inflammatory infiltrates in inflammatory myopathies," *The American Journal of Pathology*, vol. 159, no. 4, pp. 1263–1273, 2001.

[59] H. Wiendl, A. Lautwein, M. Mitsdörffer et al., "Antigen processing and presentation in human muscle: cathepsin S is critical for MHC class II expression and upregulated in inflammatory myopathies," *Journal of Neuroimmunology*, vol. 138, no. 1-2, pp. 132–143, 2003.

[60] I. Ferrer, B. Martín, J. G. Castaño, J. J. Lucas, D. Moreno, and M. Olivé, "Proteasomal expression, induction of immunoproteasome subunits, and local MHC class I presentation in myofibrillar myopathy and inclusion body myositis," *Journal of Neuropathology and Experimental Neurology*, vol. 63, no. 5, pp. 484–498, 2004.

[61] A. Saito, I. Higuchi, M. Nakagawa et al., "An overexpression of fibroblast growth factor (FGF) and FGF receptor 4 in a severe clinical phenotype of facioscapulohumeral muscular dystrophy," *Muscle & Nerve*, vol. 23, pp. 490–497, 2000.

[62] M. Cassano, A. Dellavalle, F. S. Tedesco et al., "Alpha sarcoglycan is required for FGF-dependent myogenic progenitor cell proliferation in vitro and in vivo," *Development*, vol. 138, no. 20, pp. 4523–4533, 2011.

[63] C. Bocelli-Tyndall, P. Zajac, N. di Maggio et al., "Fibroblast growth factor 2 and platelet-derived growth factor, but not platelet lysate, induce proliferation-dependent, functional class II major histocompatibility complex antigen in human mesenchymal stem cells," *Arthritis & Rheumatism*, vol. 62, no. 12, pp. 3815–3825, 2010.

[64] H. Wiendl, L. Behrens, S. Maier, M. A. Johnson, E. H. Weiss, and R. Hohlfeld, "Muscle fibers in inflammatory myopathies and cultured myoblasts express the nonclassical major histocompatibility antigen HLA-G," *Annals of Neurology*, vol. 48, pp. 679–684, 2000.

[65] N. Rouas-Freiss, R. M. Gonçalves, C. Menier, J. Dausset, and E. D. Carosella, "Direct evidence to support the role of HLA-G in protecting the fetus from maternal uterine natural killer cytolysis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 21, pp. 11520–11525, 1997.

[66] E. D. Carosella, P. Moreau, S. Aractingi, and N. Rouas-Freiss, "HLA-G: a shield against inflammatory aggression," *Trends in Immunology*, vol. 22, no. 10, pp. 553–555, 2001.

[67] L. Behrens, M. Kerschensteiner, T. Misgeld, N. Goebels, H. Wekerle, and R. Hohlfeld, "Human muscle cells express a functional costimulatory molecule distinct from B7.1 (CD80) and B7.2 (CD86) in vitro and in inflammatory lesions," *The Journal of Immunology*, vol. 161, pp. 5943–5951, 1998.

[68] K. Murata and M. C. Dalakas, "Expression of the costimulatory molecule BB-1, the ligands CTLA-4 and CD28, and their mRNA in inflammatory myopathies," *The American Journal of Pathology*, vol. 155, no. 2, pp. 453–460, 1999.

[69] K. M. Fries, G. D. Sempowski, A. A. Gaspari, T. Blieden, R. J. Looney, and R. P. Phipps, "CD40 expression by human fibroblasts," *Clinical Immunology and Immunopathology*, vol. 77, no. 1, pp. 42–51, 1995.

[70] K. Karmann, C. C. W. Hughes, J. Schechner, W. C. Fanslow, and J. S. Pober, "CD40 on human endothelial cells: Inducibility by cytokines and functional regulation of adhesion molecule expression," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 10, pp. 4342–4346, 1995.

[71] T. Sugiura, Y. Kawaguchi, M. Harigai et al., "Increased CD40 expression on muscle cells of polymyositis and dermatomyositis: role of CD40-CD40 ligand interaction in IL-6, IL-8, IL-15, and monocyte chemoattractant protein-1 production," *Journal of Immunology*, vol. 164, no. 12, pp. 6593–6600, 2000.

[72] E. Bartoccioni, S. Gallucci, F. Scuderi et al., "MHC class I, MHC class II and intercellular adhesion molecule-1 (ICAM-1) expression in inflammatory myopathies," *Clinical and Experimental Immunology*, vol. 95, no. 1, pp. 166–172, 1994.

[73] D. S. Tews and H. H. Goebel, "Expression of cell adhesion molecules in inflammatory myopathies," *Journal of Neuroimmunology*, vol. 59, no. 1-2, pp. 185–194, 1995.

[74] W. Malorni, F. Iosi, D. Zarcone et al., "Role of adhesion molecules in the mechanism of non-MHC (major histocompatibility complex) restricted cell-mediated cytotoxicity," *Scanning Microscopy*, vol. 7, no. 1, pp. 323–332, 1993.

[75] L. M. Francisco, P. T. Sage, and A. H. Sharpe, "The PD-1 pathway in tolerance and autoimmunity," *Immunological Reviews*, vol. 236, no. 1, pp. 219–242, 2010.

[76] K. English, R. Tonlorenzi, G. Cossu, and K. J. Wood, "Mesoangioblasts suppress T cell proliferation through IDO and PGE-2-dependent pathways," *Stem Cells and Development*, vol. 22, no. 3, pp. 512–523, 2013.

[77] H. Wiendl, M. Mitsdoerffer, D. Schneider et al., "Human muscle cells express a B7-related molecule, B7-H1, with strong negative immune regulatory potential: a novel mechanism of counterbalancing the immune attack in idiopathic inflammatory myopathies," *FASEB Journal*, vol. 17, no. 13, pp. 1892–1894, 2003.

[78] P. F. Lesault, M. Theret, M. Magnan et al., "Macrophages improve survival, proliferation and migration of engrafted myogenic precursor cells into MDX skeletal muscle," *PLoS ONE*, vol. 7, no. 10, Article ID e46698, 2012.

[79] K. Lolmede, L. Campana, M. Vezzoli et al., "Inflammatory and alternatively activated human macrophages attract vessel-associated stem cells, relying on separate HMGB1- and MMP-9-dependent pathways," *Journal of Leukocyte Biology*, vol. 85, no. 5, pp. 779–787, 2009.

[80] Y. Torrente, E. El Fahime, N. J. Caron et al., "Tumor necrosis factor-α (TNF-α) stimulates chemotactic response in mouse

myogenic cells," *Cell Transplantation*, vol. 12, no. 1, pp. 91–100, 2003.

[81] G. Page, G. Chevrel, and P. Miossec, "Anatomic localization of immature and mature dendritic cell subsets in dermatomyositis and polymyositis: interaction with chemokines and Th1 cytokine-producing cells," *Arthritis and Rheumatism*, vol. 50, no. 1, pp. 199–208, 2004.

[82] N. Schwab, A. Waschbisch, B. Wrobel, H. Lochmüller, C. Sommer, and H. Wiendl, "Human myoblasts modulate the function of antigen-presenting cells," *Journal of Neuroimmunology*, vol. 200, no. 1-2, pp. 62–70, 2008.

[83] O. Li, K. English, R. Tonlorenzi, G. Cossu, F. S. Tedesco, and K. J. Wood, "Human iPSC-derived mesoangioblasts, like their tissue-derived counterparts, suppress T cell proliferation through IDO- and PGE-2-dependent pathways," *F1000 Research*, vol. 2, article 24, 2013.

[84] M. Noviello, F. S. Tedesco, A. Bondanza et al., "Inflammation converts human mesoangioblasts into targets of alloreactive immune responses: implications for allogeneic cell therapy of DMD," *Molecular therapy*, 2014.

[85] H. X. Nguyen and J. G. Tidball, "Interactions between neutrophils and macrophages promote macrophage killing of rat muscle cells *in vitro*," *The Journal of Physiology*, vol. 547, no. 1, pp. 125–132, 2003.

[86] C. Noone, A. Kihm, K. English, S. O'Dea, and B. P. Mahon, "IFN-γ stimulated human umbilical-tissue-derived cells potently suppress NK activation and resist NK-mediated cytotoxicity in vitro," *Stem Cells and Development*, vol. 22, no. 22, pp. 3003–3014, 2013.

[87] T. A. Partridge, "The mdx mouse model as a surrogate for Duchenne muscular dystrophy," *FEBS Journal*, vol. 280, no. 17, pp. 4177–4186, 2013.

[88] M. Dezawa, H. Ishikawa, Y. Itokazu et al., "Bone marrow stromal cells generate muscle cells and repair muscle degeneration," *Science*, vol. 309, no. 5732, pp. 314–317, 2005.

[89] A. Farini, M. Meregalli, M. Belicchi et al., "T and B lymphocyte depletion has a marked effect on the fibrosis of dystrophic skeletal muscles in the scid/mdx mouse," *Journal of Pathology*, vol. 213, no. 2, pp. 229–238, 2007.

[90] R. W. Arpke, R. Darabi, T. L. Mader et al., "A new immuno-, dystrophin-deficient model, the NSG-mdx4Cv mouse, provides evidence for functional improvement following allogeneic satellite cell transplantation," *Stem Cells*, vol. 31, no. 8, pp. 1611–1620, 2013.

[91] D. Vallese, E. Negroni, S. Duguez et al., "The Rag2(-)Il2rb(-) Dmd(-) mouse: a novel dystrophic and immunodeficient model to assess innovating therapeutic strategies for muscular dystrophies, M," *Molecular Therapy*, vol. 21, p. 1950, 2013.

[92] F. S. Tedesco, M. F. M. Gerli, L. Perani et al., "Transplantation of genetically corrected human iPSC-derived progenitors in mice with limb-girdle muscular dystrophy," *Science Translational Medicine*, vol. 4, no. 140, Article ID 140ra89, 2012.

[93] J. Díaz-Manera, T. Touvier, A. Dellavalle et al., "Partial dysferlin reconstitution by adult murine mesoangioblasts is sufficient for full functional recovery in a murine model of dysferlinopathy," *Cell Death & Disease*, vol. 1, no. 8, article e61, 2010.

[94] A. Filareto, R. Darabi, and R. C. Perlingeiro, "Engraftment of ES-derived myogenic progenitors in a severe mouse model of muscular dystrophy," *Journal of Stem Cell Research & Therapy*, vol. 10, no. 1, article S10-001, 2012.

[95] J. N. Kornegay, S. M. Tuler, D. M. Miller, and D. C. Levesque, "Muscular dystrophy in a litter of golden retriever dogs," *Muscle and Nerve*, vol. 11, no. 10, pp. 1056–1064, 1988.

[96] N. J. H. Sharp, J. N. Kornegay, S. D. van Camp et al., "An error in dystrophin mRNA processing in golden retriever muscular dystrophy, an animal homologue of Duchenne muscular dystrophy," *Genomics*, vol. 13, no. 1, pp. 115–121, 1992.

[97] Y. Shimatsu, K. Katagiri, T. Furuta et al., "Canine X-linked muscular dystrophy in Japan (CXMDJ)," *Experimental Animals*, vol. 52, no. 2, pp. 93–97, 2003.

[98] M. Sampaolesi, S. Blot, G. D'Antona et al., "Mesoangioblast stem cells ameliorate muscle function in dystrophic dogs," *Nature*, vol. 444, no. 7119, pp. 574–579, 2006.

[99] Y. Nitahara-Kasahara, H. Hayashita-Kinoh, S. Ohshima-Hosoyama et al., "Long-term engraftment of multipotent mesenchymal stromal cells that differentiate to form myogenic cells in dogs with duchenne muscular dystrophy," *Molecular Therapy*, vol. 20, no. 1, pp. 168–177, 2012.

[100] K. Rouger, T. Larcher, L. Dubreil et al., "Systemic delivery of allogenic muscle stem cells induces long-term muscle repair and clinical efficacy in duchenne muscular dystrophy dogs," *The American Journal of Pathology*, vol. 179, no. 5, pp. 2501–2518, 2011.

[101] N. Klymiuk, A. Blutke, A. Graf et al., "Dystrophin-deficient pigs provide new insights into the hierarchy of physiological derangements of dystrophic muscle," *Human Molecular Genetics*, vol. 22, pp. 4368–4382, 2013.

[102] D. Skuk, M. Goulet, B. Roy, and J. P. Tremblay, "Efficacy of myoblast transplantation in nonhuman primates following simple intramuscular cell injections: toward defining strategies applicable to humans," *Experimental Neurology*, vol. 175, no. 1, pp. 112–126, 2002.

[103] S. Noguchi, T. Tsukahara, M. Fujita et al., "cDNA microarray analysis of individual Duchenne muscular dystrophy patients," *Human Molecular Genetics*, vol. 12, no. 6, pp. 595–600, 2003.

[104] M. Pescatori, A. Broccolini, C. Minetti et al., "Gene expression profiling in the early phases of DMD: a constant molecular signature characterizes DMD muscle from early postnatal life throughout disease progression," *FASEB Journal*, vol. 21, no. 4, pp. 1210–1226, 2007.

[105] K. English and K. J. Wood, "Mesenchymal stromal cells in transplantation rejection and tolerance," *Cold Spring Harbor Perspectives in Medicine*, vol. 3, no. 5, Article ID a015560, 2013.

[106] E. J. Gang, R. Darabi, D. Bosnakovski et al., "Engraftment of mesenchymal stem cells into dystrophin-deficient mice is not accompanied by functional recovery," *Experimental Cell Research*, vol. 315, no. 15, pp. 2624–2636, 2009.

[107] M. Guttinger, E. Tafi, M. Battaglia, M. Coletta, and G. Cossu, "Allogeneic mesoangioblasts give rise to alpha-sarcoglycan expressing fibers when transplanted into dystrophic mice," *Experimental Cell Research*, vol. 312, no. 19, pp. 3872–3879, 2006.

[108] A. J. Nauta, G. Westerhuis, A. B. Kruisselbrink, E. G. A. Lurvink, R. Willemze, and W. E. Fibbe, "Donor-derived mesenchymal stem cells are immunogenic in an allogeneic host and stimulate donor graft rejection in a nonmyeloablative setting," *Blood*, vol. 108, no. 6, pp. 2114–2120, 2006.

[109] P. Gregorevic, J. M. Allen, E. Minami et al., "rAAV6-microdystrophin preserves muscle function and extends lifespan in severely dystrophic mice," *Nature Medicine*, vol. 12, no. 7, pp. 787–789, 2006.

[110] F. Mingozzi, J. J. Meulenberg, D. J. Hui et al., "AAV-1-mediated gene transfer to skeletal muscle in humans results in dose-dependent activation of capsid-specific T cells," *Blood*, vol. 114, no. 10, pp. 2077–2086, 2009.

[111] J. R. Mendell, K. Campbell, L. Rodino-Klapac et al., "Dystrophin immunity in Duchenne's muscular dystrophy," *The New England Journal of Medicine*, vol. 363, no. 15, pp. 1429–1437, 2010.

[112] K. J. Wood, A. Bushell, and J. Hester, "Regulatory immune cells in transplantation," *Nature Reviews Immunology*, vol. 12, no. 6, pp. 417–430, 2012.

[113] F. Mingozzi, Y. Liu, E. Dobrzynski et al., "Induction of immune tolerance to coagulation factor IX antigen by in vivo hepatic gene transfer," *Journal of Clinical Investigation*, vol. 111, no. 9, pp. 1347–1356, 2003.

[114] A. Annoni, A. Cantore, D. P. Valle et al., "Liver gene therapy by lentiviral vectors reverses anti-factor IX pre-existing immunity in haemophilic mice," *EMBO Molecular Medicine*, vol. 5, no. 11, pp. 1684–1697, 2013.

[115] D. Burzyn, W. Kuswanto, D. Kolodin et al., "A special population of regulatory T cells potentiates muscle repair," *Cell*, vol. 155, pp. 1282–1295, 2013.

[116] C. M. Kelly, S. V. Precious, C. Scherf et al., "Neonatal desensitization allows long-term survival of neural xenotransplants without immunosuppression," *Nature Methods*, vol. 6, no. 4, pp. 271–273, 2009.

[117] K. Takenaka, T. K. Prasolava, J. C. Y. Wang et al., "Polymorphism in Sirpa modulates engraftment of human hematopoietic stem cells," *Nature Immunology*, vol. 8, no. 12, pp. 1313–1323, 2007.

[118] S. Jaiswal, C. H. M. Jamieson, W. W. Pang et al., "CD47 is upregulated on circulating hematopoietic stem cells and leukemia cells to avoid phagocytosis," *Cell*, vol. 138, no. 2, pp. 271–285, 2009.

[119] T. A. Partridge, J. E. Morgan, G. R. Coulton, E. P. Hoffman, and L. M. Kunkel, "Conversion of mdx myofibres from dystrophin-negative to-positive by injection of normal myoblasts," *Nature*, vol. 337, pp. 176–179, 1989.

[120] T. Partridge, "The current status of myoblast transfer," *Neurological Sciences*, vol. 21, no. 9, pp. S939–S942, 2000.

[121] J. P. Tremblay, F. Malouin, R. Roy et al., "Results of a triple blind clinical study of myoblast transplantations without immunosuppressive treatment in young boys with Duchenne muscular dystrophy," *Cell Transplantation*, vol. 2, no. 2, pp. 99–112, 1993.

[122] B. Guerette, I. Asselin, J. T. Vilquin, R. Roy, and J. P. Tremblay, "Lymphocyte infiltration following allo- and xenomyoblast transplantation in mdx mice," *Muscle & Nerve*, vol. 18, no. 1, pp. 39–51, 1995.

[123] I. Kinoshita, J.-T. Vilquin, B. Guerette, I. Asselin, R. Roy, and J. P. Tremblay, "Very efficient myoblast allotransplantation in mice under FK506 immunosuppression," *Muscle and Nerve*, vol. 17, no. 12, pp. 1407–1415, 1994.

[124] D. Skuk, N. Caron, M. Goulet, B. Roy, F. Espinosa, and J. P. Tremblay, "Dynamics of the early immune cellular reactions after myogenic cell transplantation," *Cell Transplantation*, vol. 11, no. 7, pp. 671–681, 2002.

[125] J. R. Beauchamp, J. E. Morgan, C. N. Pagel, and T. A. Partridge, "Dynamics of myoblast transplantation reveal a discrete minority of precursors with stem cell-like properties as the myogenic source," *The Journal of Cell Biology*, vol. 144, no. 6, pp. 1113–1122, 1999.

[126] L. M. Sammels, E. Bosio, C. T. Fragall, M. D. Grounds, N. van Rooijen, and M. W. Beilharz, "Innate inflammatory cells are not responsible for early death of donor myoblasts after myoblast transfer therapy," *Transplantation*, vol. 77, no. 12, pp. 1790–1797, 2004.

[127] D. Skuk, M. Paradis, M. Goulet, and J. P. Tremblay, "Ischemic central necrosis in pockets of transplanted myoblasts in nonhuman primates: implications for cell-transplantation strategies," *Transplantation*, vol. 84, no. 10, pp. 1307–1315, 2007.

[128] D. Skuk, M. Goulet, B. Roy et al., "Dystrophin expression in muscles of Duchenne muscular dystrophy patients after high-density injections of normal myogenic cells," *Journal of Neuropathology and Experimental Neurology*, vol. 65, no. 4, pp. 371–386, 2006.

[129] S. Perie, C. Trollet, V. Mouly et al., "Autologous myoblast transplantation for oculopharyngeal muscular dystrophy: a phase i/iia clinical study," *Molecular Therapy*, vol. 22, no. 1, pp. 219–225, 2014.

[130] O. Benveniste, M. Guiguet, J. Freebody et al., "Long-term observational study of sporadic inclusion body myositis," *Brain*, vol. 134, no. 11, pp. 3176–3184, 2011.

[131] J. R. Mendell, L. R. Rodino-Klapac, X. Q. Rosales et al., "Sustained alpha-sarcoglycan gene expression after gene transfer in limb-girdle muscular dystrophy, type 2D," *Annals of Neurology*, vol. 68, no. 5, pp. 629–638, 2010.

[132] Y. Torrente, M. Belicchi, C. Marchesi et al., "Autologous transplantation of muscle-derived CD133+ stem cells in Duchenne muscle patients," *Cell Transplantation*, vol. 16, no. 6, pp. 563–577, 2007.

[133] A. Lerario, S. Bonfiglio, M. Sormani et al., "Quantitative muscle strength assessment in duchenne muscular dystrophy: longitudinal study and correlation with functional measures," *BMC Neurology*, vol. 12, article 91, 2012.

[134] M. Giannotta, S. Benedetti, F. S. Tedesco et al., "Targeting endothelial junctional adhesion molecule-A/EPAC/Rap-1 axis as a novel strategy to increase stem cell engraftment in dystrophic muscles," *EMBO Molecular Medicine*, vol. 66, no. 2, pp. 239–258, 2014.

[135] F. S. Tedesco, H. Hoshiya, G. D'Antona et al., "Stem cell-mediated transfer of a human artificial chromosome ameliorates muscular dystrophy," *Science Translational Medicine*, vol. 3, article 96ra78, 2011.

[136] M. F. Gerli, S. M. Maffioletti, Q. Millet, and F. S. Tedesco, "Transplantation of Induced Pluripotent Stem Cell-derived Mesoangioblast-like Myogenic Progenitors in Mouse Models of Muscle Regeneration," *Journal of Visualized Experiments*, vol. 83, Article ID e50532, 2014.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 438675, 12 pages
http://dx.doi.org/10.1155/2014/438675

Review Article

From Innate to Adaptive Immune Response in Muscular Dystrophies and Skeletal Muscle Regeneration: The Role of Lymphocytes

Luca Madaro[1] **and Marina Bouché**[2]

[1] *IRCCS Fondazione Santa Lucia, Via del Fosso di Fiorano 64, 00143 Rome, Italy*
[2] *DAHFMO, Unit of Histology and Medical Embryology, Sapienza University of Rome, Via Antonio Scarpa 14, 00161 Rome, Italy*

Correspondence should be addressed to Marina Bouché; marina.bouche@uniroma1.it

Received 15 February 2014; Accepted 2 May 2014; Published 16 June 2014

Academic Editor: Pura Muñoz-Cánoves

Skeletal muscle is able to restore contractile functionality after injury thanks to its ability to regenerate. Following muscle necrosis, debris is removed by macrophages, and muscle satellite cells (MuSCs), the muscle stem cells, are activated and subsequently proliferate, migrate, and form muscle fibers restoring muscle functionality. In most muscle dystrophies (MDs), MuSCs fail to properly proliferate, differentiate, or replenish the stem cell compartment, leading to fibrotic deposition. However, besides MuSCs, interstitial nonmyogenic cells and inflammatory cells also play a key role in orchestrating muscle repair. A complete understanding of the complexity of these mechanisms should allow the design of interventions to attenuate MDs pathology without disrupting regenerative processes. In this review we will focus on the contribution of immune cells in the onset and progression of MDs, with particular emphasis on Duchenne muscular dystrophy (DMD). We will briefly summarize the current knowledge and recent advances made in our understanding of the involvement of different innate immune cells in MDs and will move on to critically evaluate the possible role of cell populations within the acquired immune response. Revisiting previous observations in the light of recent evidence will likely change our current view of the onset and progression of the disease.

1. Background

Only a small number of immune cells reside within intact skeletal muscle, but they are recruited during injury and play important roles in the regeneration process, critically contributing to its resolution. Upon injury, immune cells rapidly infiltrate the muscle to remove necrotic tissue and secrete soluble factors that serve initially to activate muscle satellite cells (MuSCs) [1–3]. As such, immune cells constitute a transient local environment for MuSCs. Satellite cells and immune cells attract one another through chemokines (chemoattraction). Satellite cells have been demonstrated to secrete a panel of proinflammatory cytokines, such as IL-1, IL-6, and TNF-α to facilitate immune cell infiltration and function [4, 5]. In turn, immune cells secrete a wealth of diffusible factors, such as growth factors, IL-6, globular adiponectin, extracellular matrix (ECM) components, and ECM remodeling MMPs. These diffusible factors generate ECM chemoattractive fragments, which help satellite cells escape from the basal lamina of myofibers, and promote satellite cell proliferation [6]. In addition, cell-to-cell contact between immune and satellite cells protects satellite cells from apoptosis [7]. All these events must be timely regulated and alterations in quality, amount, and time lead to impaired regeneration, increased muscle wasting, and deposition of fibrotic tissue, as it occurs in muscle aging or in muscular dystrophies, such as Duchenne muscular dystrophy (DMD) [8, 9]. DMD is a lethal X-linked genetic disorder caused by deficiency of dystrophin, a critical component of the dystrophin glycoprotein complex (DGC), acting as a link between the cytoskeleton and extracellular matrix in skeletal and cardiac muscles [10]. A consequence of the DGC inefficiency is muscle fragility, contraction-induced damage, necrosis, and inflammation. Although satellite cells compensate for muscle fiber loss in the early stages

of disease, eventually these progenitors become exhausted [11]. Moreover, aberrant intracellular signalling cascades that regulate both inflammatory and immune processes contribute substantially to the degenerative process. As a result, fibrous and fatty connective tissue overtakes the functional myofibers. These changes culminate in progressive muscle wasting, with the majority of patients being wheelchair-bound in their early teens, succumbing to cardiac/respiratory failure in their twenties [12]. Among the animal models of DMD, the *mdx* mouse model is the best characterized. It lacks dystrophin expression, and, though with a milder phenotype, exhibits extensive limb muscle degeneration and inflammation, as well as myocardial lesions [13–15]. Available data sets, although limited and not comprehensive, suggest that early immune cell infiltration in DMD patients and *mdx* mice represent an important, but underappreciated, aspect of dystrophic muscle pathology. In fact, although lack of dystrophin makes myofibers susceptible to fragility and degeneration when contracting, this mechanical defect hypothesis for dystrophic muscle death has been unable to explain many aspects of the pathophysiology of DMD and emerging clues attribute an active role to the immune response in these events [16]. From a therapeutic point of view, a clear understanding of the cell populations and of the mechanisms involved in the inflammatory response in DMD may allow the design of valuable anti-inflammatory therapeutic strategies to ameliorate muscular dystrophy. Although blunting inflammation would not restore the primary defect, the emerging clue is that multiple strategies, addressing different aspects of the pathology, which may eventually converge, may be successful.

2. Innate Immune Response in Muscular Dystrophies

As a consequence of the dystrophin-null myofibers fragility, DMD muscle is characterized by continuous cycles of myofibers necrosis and repair. Myofibers undergoing degeneration/necrosis, independently of the injury insult, release Th1 inflammatory stimuli, which recruit neutrophils and monocytes/macrophages required to clear cell debris, followed by a Th2 immune response which promotes muscle healing [17]. Much of the current information about the role of immune cell populations of the innate response in muscle repair comes from studies on healthy regenerating muscle [1, 18–20]. Although many similarities were found between acute and chronic muscle injury, the kinetics, quality, and outcome diversify in many aspects [21, 22].

2.1. Neutrophils. In acute injury, neutrophils, identified as Ly6C+/F4/80− cells, are the first cells to invade injured muscle, followed by macrophages. In acutely injured muscle in mice, they begin to appear at elevated numbers within 2 h of muscle damage, typically peaking in concentration between 6 and 24 h after injury and then rapidly decline in numbers [23]. Their function mostly involves phagocytic activity to remove debris but also release of TNFα, as a Th1 stimuli, and production of myeloperoxidase (MPO), inducing muscle membrane damage and increasing macrophage proinflammatory activity [24–26]. As in acute injury, neutrophils, together with macrophages, invade *mdx* dystrophic muscle as early as 2 weeks of age [24]. Indeed, initial muscle injury and membrane lysis are caused by superoxide production mediated by these early infiltrating neutrophils. Previous studies demonstrated that anti-GR1 antibody-mediated depletion of neutrophils, starting at age 19 days, significantly reduces muscle necrosis at age ~21 days and subsequent regeneration at age ~28 days [25]. However, GR-1 is highly expressed in neutrophils, but it is also expressed in macrophages [27, 28], so whether the observed phenotype was specifically related to neutrophils depletion is not as clear. In any case, although neutrophils activity appears to overlap with that of M1 macrophages (see below), since they are one of the earliest infiltrating cell types, they might promote initial muscle lesion, playing an important role in the early onset of the pathology.

2.2. Macrophages. A comprehensive survey of the current knowledge on macrophage involvement in MDs and muscle regeneration is beyond the scope of this review. The reader is referred to many systematic reviews already available on this topic [16, 21, 29]. Briefly, two subpopulations of macrophages have been identified in regenerating muscle tissue that may influence muscle degeneration and regeneration depending on the proportion of these cells present [18, 30]. As a simplistic point of view, the M1 population are proinflammatory, characterized by the expression of iNOS and secretion of proinflammatory cytokines (e.g., TNFα, IL-1β, and IL-6), and promote muscle cell lysis; by contrast, the M2 population is characterized by the expression of arginase-1, CD163, and CD206 mannose receptor (usually in noninflammatory, repair conditions) and/or anti-inflammatory cytokines (e.g., IL-10) [19, 21, 31, 32]. They are believed to enhance muscle regeneration, by inducing satellite cell proliferation [19]. However, beyond this broad definition, mainly determined from *in vitro* polarization experiments, macrophages exhibit a wide variety of intermediate phenotype, including M2b and M2c, M1 and M2 being the extremes of a continuum in activation states. M2b macrophages are known to release large amounts of IL-10, which promotes the proliferation of nonmyeloid cells, although, like M1 macrophages, they can also release proinflammatory cytokines, such as IL-1β and TNFα. IL-10 can also induce M2c macrophages, which have anti-inflammatory functions [33]. However, it has been recently highlighted that several markers should be examined at the cellular level in order to properly assess the macrophage inflammatory state *in vivo*, since the population remains heterogeneous [34]. While in acute muscle injury the sequential waves of M1, followed by M2 macrophage invasion, leads to resolution of inflammation and efficient muscle repair, in muscular dystrophies, repetitive cycles of myofiber degeneration lead to muscle invasion by M1 macrophages

together with M2a macrophages, which may reduce the cytotoxic activity of M1 macrophages [35]. Therefore, the inflammatory *milieu* in dystrophic muscle is similar, but not the same as found in acute injury. Subsequent invasion of dystrophic muscle by M2c macrophages is associated with progression to the regenerative phase in pathophysiology. However, the number of M2 macrophages declines upon resolution of the damage in acute-injured muscle, while in *mdx* muscle their number increases with age, and promotes fibrosis [36]. Thus, increased and persistent presence of macrophages modifies the intensity, duration, and interactions of the different released factors, leading to increased myofibers necrosis, ECM accumulation, and replacement of muscle with fibrotic and fat tissue. Early studies showed that depletion of macrophages from *mdx* mice before the onset of histopathology caused great reduction in muscle pathology in 4-week-old mice [37], reinforcing the notion that macrophages induce muscle lesions, along with clearing debris. However, given that myofiber degeneration occurs in the absence of dystrophin, depletion of macrophages might not be as beneficial in long term, as it would prevent removal of debris. Indeed, whether or not phagocytosis is required for proper muscle repair is still debatable. Depletion of phagocytes was shown to slow cellular debris removal following freeze-injury of muscle but to have no effect on early postinjury regeneration, in terms of satellite cells activation [38]. By contrast, other studies have concluded that reducing the numbers of phagocytic leukocytes (neutrophils and monocytes/macrophages) in mice prior to toxin-induced muscle injury slows the removal of cellular debris and slows muscle regeneration, since reduced frequency of central-nucleated muscle fibers was observed [18, 39]. However, considering that, as mentioned before, phagocytic leukocytes also promote muscle damage, it could be argued that reduced number of regenerating fibers was dependent on reduced muscle damage because of the depletion of leukocytes. In this context, a recent study by Mounier et al. showed that inhibiting phagocytic activity in macrophages prevents macrophage skewing from a pro- to anti-inflammatory phenotype at the time of resolution of inflammation, in regenerating healthy muscle, suggesting that phagocytosis is required not only to clear debris, but also to promote resolution of the damage [34].

Although these studies are related to healthy regenerating muscle, deciphering the mechanisms regulating macrophages activation and plasticity should greatly facilitate the design of novel therapeutic strategies to modify the dystrophic muscle environment, reduce muscle damage, and increase muscle repair.

2.3. Mast Cells. Very little is known about mast cell involvement in muscular dystrophies. Nevertheless, mast cell proliferation and degranulation are often observed in areas of grouped fiber necrosis [40]. Mast cell degranulation involves the release of histamine and cytokines (including TNFα), which contribute to a pro-inflammatory environment that promotes muscle necrosis. In addition, proteases released during mast cell degranulation induce membrane lysis of nearby cells promoting local areas of ischemia [41]. Indeed, treatment of *mdx* mice prior to disease onset (age 19 days) with sodium cromoglycate (cromolyn), a blocker of mast cell degranulation, results in ~59% decrease in cumulative TA muscle damage by age 28 days [42]. Further studies are needed to gain insight into the mechanisms that regulate the microenvironment and ischemic conditions that result in fiber necrosis in muscular dystrophies. In a mouse model of arthritis, an inflammatory disease not related to muscle, mast cell-mediated increase of vascular permeability has been shown to be involved in the recruitment of inflammatory cells into the site of inflammation [43]. Interestingly, it has been recently shown that mast cells deficiency in a mouse model of polymyositis results in reduced susceptibility to C-protein-induced myositis, as compared with WT [44]. The reduced susceptibility to C-protein-induced myositis was associated with a reduced number of macrophages and CD8+ T cells, mainly due to reduced vascular permeability. The observed reduced susceptibility was restored by reconstitution of mast cells, highlighting the crucial role of these cells in promoting massive inflammatory cells infiltration [44]. Polymyositis (PM) belongs to the class of idiopathic inflammatory myopathies, IIM, which also includes adult and juvenile dermatomyositis (DM), myositis, and inclusion body myositis (IBM), a heterogeneous group of acquired disorders characterized by chronic inflammation of striated muscle leading to predominantly proximal muscle weakness. Although the precise pathogenesis is unknown, many evidences support their autoimmune basis and they likely result from immune-mediated processes initiated by environmental factors in genetically susceptible individuals [45, 46].

Increase in vascular permeability is certainly one of the events contributing to the immune cell infiltration in dystrophic muscle, as well. These aspects have been poorly addressed in muscular dystrophy, as only recently the disease was recognized as an "inflammatory" disease; investigating the disease from this new perspective may give unexpected insights into the mechanisms regulating its onset and progression.

2.4. Eosinophils. Eosinophils are generally associated with immune responses to infection by parasites or with allergic reactions [47]. However, they can play a significant role in the cellular immune response, in which their involvement appears to be tightly coupled both temporally and functionally to T lymphocyte activity; indeed, they are activated by the Th2 cytokine IL-5 [48, 49]. Although eosinophilia occurs only rarely and inexplicably in muscle disease, eosinophil invasion was found in both DMD and *mdx* dystrophies [50, 51]. Previous studies observed that eosinophils increased within *mdx* dystrophic muscle at about 4 weeks of age, together with cytotoxic T cells invasion, and, although their number decreases during the regenerative phase, their concentration remains higher at 30–32 weeks of age, as compared to healthy muscle of age-matched wild type mice, depending on the muscle examined [51]. Interestingly, transplantation of splenocytes derived from *mdx* mice into irradiated WT

animal induced an increased number of eosinophils in muscle, as compared to irradiated control mice, suggesting that eosinophils invasion is dependent on lymphocytes activity [51]. Indeed, prednisone treatment (the major drug used in DMD patients) reduces eosinophil infiltration [52]. Moreover, despite the original expectation that eosinophilia in dystrophic muscle was a nonspecific consequence of the Th2 inflammatory environment, eosinophils were shown to modulate injury and regeneration. In particular, genetic ablation of major basic protein-1 (MBP-1), a cytolytic protein expressed by eosinophils, caused an increase in the numbers of cytotoxic T cells in dystrophic muscle [50]. That finding is significant to *mdx* dystrophy because a Th1-driven cellular immune response in which cytotoxic T cells promote apoptosis of *mdx* muscle fibers is an early feature of the disease that is attenuated as muscle regeneration begins. Thus, eosinophils may mediate the regeneration of dystrophic muscle by promoting the transition from Th1 to Th2 inflammatory environment. An interesting insight on the possible role of eosinophils in promoting muscle regeneration was recently suggested by Heredia and colleagues [17]. They showed that eosinophils invade acute injured muscle very early, even before neutrophils invasion. This observation is in contrast to other studies which showed, using the same injury model, that eosinophils are exceeding scarce in muscle following toxin injection and they appear to increase after other inflammatory cell populations [34]. However, using the eosinophil-deficient ΔdblGATA1 mouse model, which presents a 90% reduction in eosinophils, Heredia et al. observed persistent and unresolved muscle damage after acute injury. The authors identified IL-4 as the key cytokine produced by eosinophils, whose lack is known to prevent muscle regeneration [53]. Surprisingly, they demonstrate that the primary target of this cytokine produced by eosinophils at the very early stage of muscle damage is not macrophage, as expected, but fibroadipogenic precursor (FAP) cells. In fact, while lack of IL-4 receptor in macrophages did not result in regenerative defect, the lack of this receptor in FAPs is deleterious and leads to fat deposition by promoting their differentiation into adipocytes. FAPs are recently identified multipotent mesenchymal cells residing in skeletal muscle interstitium, who represent a critical component of the cellular niche required for effective satellite cell-mediated regeneration [54–57]. Thus, eosinophils may form a transitional niche for proliferating FAPs via secretion of IL-4; in the presence of IL-4, FAPs proliferate as fibroblasts to support myogenesis [17]. So far, whether these observations are relevant to muscular dystrophy is a matter of speculation; it can be hypothesized that, in dystrophic muscle, the eosinophils promote the transition from a Th1 to a Th2 inflammatory environment, which would in turn promote FAPs proliferation as fibroblasts to support myogenesis. On the other hand, persistence of eosinophils within the dystrophic muscle would sustain fibroblast proliferation, which promotes fibrotic tissue deposition, accelerating the clinical decline of the disease [36]. Thus, further studies are needed to gain insight into the role of eosinophils in the pathophysiology of muscular dystrophy and to verify whether manipulating eosinophils activity would be therapeutically beneficial.

3. Lymphocytes and the Adaptive Immune Response in Muscle Repair

3.1. T-Cell Response in DMD. It is widely believed that lymphocytes do not play a relevant role in healthy regenerating muscle, due to the inability of skeletal muscle to activate a T-cell response. Neither MHC class I nor class II molecules has been detected on muscle fibers from healthy muscle tissues [58, 59]. By contrast, appearance of MHC class I and/or II was observed in muscle tissue of patients with idiopathic inflammatory myopathies (IIM), where an autoimmune pathogenesis is now recognized, but also in regenerating fibers of patients with DMD [58]. These findings, together with the observation that an inflammatory *milieu* may upregulate HLA-DR and costimulatory molecules (ICOS-L and CD40), led to the idea that myoblasts can become facultative APCs in both MHCI and II-dependent immune reactions, forming functional immunological synapses with T cells [59, 60]. Indeed, T cells are found in degenerating muscle after acute injury, but their recruitment is more robust and persistent in chronic diseases, such as myositis or muscular dystrophies, where also B cells can be detected [61, 62].

The recruitment of T cells into injured muscle implies an adaptive immune response, which normally depends on antigen exposure. While this mechanism appears now conceivable in inflammatory myositis, it is less clear in regenerating muscle after acute injury or in chronic diseases such as muscular dystrophies. Many observations suggest that muscle-specific autoantigens drive T-cell expansion in IIM [46], but no such observations are available in regenerating muscle or in muscular dystrophies yet. Furthermore, whether or not T-cell infiltrate critically contributes to muscle damage in muscular dystrophies is still debatable. Persistence of T cells in dystrophic muscle may actually modulate inflammatory *milieu* and immune cells activity but may also directly interfere with muscle cell function through lymphocyte-released cytokines and chemokines [63, 64]. Indeed, it was recently shown that conditioned medium from activated T-cells, previously cultured in anti-CD3 coated dishes in the presence of IL2 [65], induced proliferation of C2C12 muscle cell, a mouse derived muscle cell line widely used as a model of myogenesis *in vitro*, and prevents myogenic differentiation, as compared to conditioned medium derived from resting T cells [66].

Very early studies correlated the reduction in T cells observed in prednisone-treated DMD patients, with reduction in muscle necrosis and fibrosis [67, 68]. Further studies identified T cells in muscles of several DMD patients, characterized by a specific T-cell receptor (TCR) rearrangement [69]. The overrepresentation of a T-cell population expressing a restricted set of TCR variable genes might indicate a selective T-cell response directed to a muscle-specific antigen. Their persistence in DMD muscle could derive from either clonal expansion or conserved antigen recognition, or from the emergence of a regulatory population. Interestingly, specific TCR arrangement, different from the one found in DMD, was initially found in the T cells invading muscle in

PM patients [70]. Since then, many studies aimed to characterize T-cell populations and their role in MDs and muscle regeneration, though the results were not as exhaustive and were sometimes contradictory.

3.2. T-Cell Response in mdx Muscle. Elevated concentrations of activated cytotoxic CD8+ and helper CD4+ T cells (Th) are present in affected muscles of *mdx* mice aged 4–8 weeks but rapidly decrease in concentration by 14 weeks of age [63]. However, no increase in these cells has been observed in axillary or inguinal lymph nodes of *mdx* mice, suggesting that their activation is occurring in muscle tissue, and not systemically [63]. CD8+ T cells are the first to invade dystrophic muscle, peaking at 4 weeks of age; their activation is generally driven by a Th1 cellular immune response to kill their target cells through perforin-mediated processes [51]. Around 2 weeks later, CD4+ T cells also invade dystrophic muscle; T helper CD4+ T cells can generally differentiate into Th effector inflammatory cells, mainly Th1 and Th2, or into regulatory T cells (Treg), both of which participate in immune responses. Th1 cells are known to support macrophage M1 polarization by producing IL-1, IL-2, TNF-α, and INF-γ, while Th2 produce IL-4, IL-13, and IL-6 sustaining the M2 macrophage polarization [71]. On the other hand, Treg cells are required for the resolution of the immune response. These cells are characterized by the expression of Foxp3 transcription factor and produce anti-inflammatory cytokines such as IL-10 [72]. They were originally described as regulators of T-cell activity but were later recognized to also regulate B cells and several other innate immune response players [73].

One of the first studies addressing the possible role of lymphocytes in *mdx* showed that antibody-mediated depletion of CD8+ or CD4+ cells in *mdx* mice, beginning at 6 days of age and continuing until the age of 4 weeks, resulted in a 75% and 61% reduction in muscle histopathology, respectively [63]. This positive outcome suggested an important role for these cells in the development of muscle lesions. In another study, *scid/mdx* mice, which are deficient in functional T and B lymphocytes, were shown to develop much less diaphragm fibrosis at 1 year of age and a decrease in activated TGFβ in skeletal muscle, compared with *mdx* mice. Improvement in muscle regeneration was also observed in these mice, but not in muscle functionality [74]. Accordingly, in *nu/nu/mdx* mice, the lack of functional T cells alone was associated with less diaphragm fibrosis at 3 months of age [75]. Altogether, these results support the pathogenic role of T cells in *mdx* muscle and reveal this lymphocyte subclass as an important source of TGFβ1. By contrast, more recently, an additional dystrophic immunodeficient mouse model was generated, the Rag2(−)Il2rb(−)Dmd(−) mouse, which lacks T, B, and NK cells, and also carries a mutant Dmd allele that prevents the production of any dystrophin isoform [76]. Although not systemically analyzed, the authors reported that there was no difference in the pathological features of the disease in these mice, compared to *mdx* mice. Indeed, previous studies have shown that thymectomy at 1 month of age, which completely depletes circulating T cells, failed to prevent diaphragm fibrosis at 6 months of age [75]. Although this result suggested that the fibrotic process is self-sustaining from a very early stage, it can be argued that depleting circulating T cells at 1 month of age was already late, as early muscle invading T cells may eventually expand within the muscle and contribute to fibrous tissue deposition. However, the possibility of muscle-specific T cells clonal expansion has been poorly investigated, so far.

T cells represent approximately 3% of all infiltrating cells in *mdx* muscle, with over half present as double-negative T cells (lacking both CD4 and CD8 expression), 8%–10% of which were recently identified as being NKT-like cells, which express both T and NK markers [77]. Interestingly, a predominant T-cell population expressing Vβ8.1/8.2 was identified within the muscle infiltrate in *mdx* mice at 1 month of age. These Vβ8.1/8.2+ cells are not generally overrepresented in circulating cells, and, therefore, it is likely that these cells are either selectively home to or expand within *mdx* muscle. These cells express high level of osteopontin (OPN), which modulates cellular immune profiles in *mdx* muscles; in fact, its ablation in *mdx* mice results in significant reduction in neutrophils, CD3+/Vβ8.1/8.2+ cells, and NKT-like cells, but not macrophages. Interestingly, an increase in CD3+/CD4+/FoxP3+ Treg cells was observed, highlighting the importance of modulating T cells subclasses in muscular dystrophy. In this context, OPN appears to be an immune modulator that specifically impacts the concentration of NKT-like cells, neutrophils, and Tregs in dystrophic muscle [77]. As a result, lack of OPN in *mdx* mice reduces intramuscular TGFβ and fibrosis in both muscle and heart at 6 months of age, and importantly, it improves muscle strength. Vβ8.1/8.2+ cells are not the only source of OPN in dystrophic muscle as other immune cells, as well as muscle tissue, which could also produce it. Thus, besides the identification of OPN as a potential therapeutic target to ameliorate inflammation and progression of muscular dystrophy, these data clearly demonstrated a crucial role of T-cell subclasses in modulating the inflammatory *milieu* in dystrophic muscle. In line with this view, treatment with the immunosuppressant drug Rapamycin ameliorates the *mdx* phenotype with a reduction of both CD4+ and CD8+ cells, but not of regulatory Foxp3+ cells [64]. In this context, we recently showed that lack of protein kinase C θ (PKCθ) in *mdx* mice prevents muscle degeneration and inflammation, thus improving muscle regeneration and, importantly, muscle performance [78]. PKCθ is a member of the PKC family, predominantly expressed in muscle, where we and others have shown that it is an upstream regulator of several intracellular pathways leading to muscle homeostasis [78–83]. Furthermore, PKCθ, which is a key regulator of T-cell activation and proliferation, is being proposed as an attractive target to prevent immune response and allore-activity [84, 85]. Indeed, it appears to be required for the development of a robust inflammatory response in vivo [86, 87]. Interestingly, PKC$\theta^{-/-}$ mice fail to develop experimental allergic encephalomyelitis, display drastically reduced lung inflammation after induction of allergic asthma, and have a significantly diminished response in experimental colitis,

and a type II collagen induced arthritis model [86, 88, 89]. Of note, PKC$\theta^{-/-}$ mice can still mount a normal protective immune response to clear viral infections, and, importantly, maintain Treg function, since PKCθ is known to inhibit Treg differentiation [90, 91]. Indeed, we showed by bone marrow transplantation experiments that the improved phenotype observed in the double mutant mdx/PKC$\theta^{-/-}$ mice is primarily due to lack of PKCθ in the hematopoietic cell compartment [78]. Thus, the possibility that lack of PKCθ in T cells may alter the T-cell subclasses within dystrophic muscle, and, in turn, the inflammatory *milieu*, including macrophage subclasses and function, is currently under investigation.

3.3. T-Cell Response in Dysferlinopathies. The involvement of lymphocytes activity appears to be a general feature in muscular dystrophies. In fact, it has been recently demonstrated that lack of functional T/B cells improves regeneration in a mouse model of dysferlinopathies [92]. These diseases belong to the so-called limb girdle muscular dystrophies (LGMDs), characterized by predominant weakness and wasting of muscles of the pelvic and shoulder girdle. Among them, LGMD-2B and Miyoshi myopathy (MM) develop due to defects in the dysferlin gene, coding for a membrane protein mainly required for vesicle traffic and membrane repair [93]. Inflammatory cells were detected in both MM and LGMD patients, scattered or organized into clusters, around necrotic fibers. Inflammatory infiltrates around vessels mainly consisted of macrophages, whereas CD4+ and CD8+ cells were found in endomysial infiltrates. Abnormal MHC-I expression was observed in degenerating/regenerating fibers usually close to inflammatory cluster cells but was absent in normal fibers [58, 59]. Indeed, the dysferlin-deficient AJ mice, one of the mouse models of dysferlinopathies, lacking functional T/B cells (AJ/SCID mice) develop a milder pathologic phenotype with increased muscle regeneration and reduced percentage of proinflammatory M1 macrophages. Importantly, these mice showed an increase in muscle force. The rescue of the phenotype was primarily due to a reduced IL-6 production by macrophages [74]. Indeed, liposome-clodronate macrophage depletion impairs IL-6 production also in control AJ mice, supporting the notion that the beneficial effect of T/B cells absence is due to a switch in macrophage phenotype and function.

3.4. The Emerging Role of Treg Cells in the Immune Response to Muscle Injury. As mentioned above, although it has long been recognized that lymphocytes invade acutely injured muscle, very little is known about their role in these conditions, mainly due to the very small number of these cells within the muscle, making their characterization difficult.

Recently, several mouse models have been generated to further understand the mechanisms underlying T cells recruitment, expansion, and persistence in acutely injured muscle.

Interestingly, it was recently shown that acute myofiber damage itself transiently activates muscle-antigen-specific $CD8^+$ T cells in draining lymph nodes, supporting the idea of a transient autoreactive immune response upon muscle injury [94]. These activated $CD8^+$ T cells transiently invade acute injured muscle, but their negative impact on muscle repair is promptly controlled by immunosuppressive cues [94]. The authors proposed that inadequate control of this $CD8^+$ T-cell response might favor the emergence of sustained autoimmune myositis. In line with this view, Young et al. showed that aberrant muscle antigen exposure is sufficient to induce myositis in a Treg deficient *milieu* [95]. They generated a FoxP3/Syt VII double mutant, in which lack of synaptotagmin VII (Syt VII, a member of synaptotagmin family of membrane-trafficking proteins) impairs membrane resealing thus exposing endogenous muscle antigens [96]. Interestingly, while Syt VII mutant mice develop a mild, self-limiting inflammatory response involving muscle, FoxP3/Syt VII double mutants develop a significant inflammatory response in muscle, histologically resembling polymyositis in humans. Taken together, these studies suggest that, although muscle injury may evoke an autoreactive immune response to endogenous muscle antigens, this reaction is blunted within muscle through local immune-suppressive Treg dependent mechanism. In support of this, a muscle specific Treg population capable of potentiating muscle repair was recently identified [72]. Following cardiotoxin (CTX) muscle injury, a Treg cell population began to accumulate at day 4 after injury, just as the myeloid cell infiltrate switched from a proinflammatory to a proregenerative phenotype. Their frequency peaked at 2 weeks after injury, accounting for about 50% of the $CD4^+$ T cells. However, while the number of CD4 T cells dropped to levels characteristic of uninjured muscle by 28 days after CTX injection, the number of Treg cells remained elevated. Interestingly, the increase in their number was not due to further recruitment, rather to clonal expansion within the muscle. Although these muscle Treg cells expressed the canonical gene signature characteristic of Treg cells, microarray gene expression analysis showed that the muscle Treg cells were closely related to Treg cells derived from visceral adipose tissue (VAT), but different from Treg cells in lymphoid organs. The authors provide a large data set of genes differentially expressed in VAT, muscle, colonic lamina propria, and prediabetic NOD pancreas derived Treg cells, supporting the notion that the Treg compartment is heterogeneous, has multiple functions, and exerts effects beyond the boundaries of the immune system [97]. Importantly, using the Foxp3-DTR mouse, in which Diphtheria Toxin is expressed under the control of the promoter of the Treg-specific Foxp3 gene, they showed that ablation of Treg cells by Diphtheria Toxin injection, after muscle injury, prevented muscle regeneration [72]. These authors identified a key molecule released by muscle Treg, responsible for the observed beneficial effect, named Amphiregulin (Areg), an EGF family growth factor. Administration of Areg in mice lacking Tregs restored efficient muscle regeneration, suggesting that this growth factor is part of fundamental cues during muscle repair. In this context, previous studies have suggested that Areg might act directly on muscle cells [98, 99]. Interestingly, a Treg population was enriched in dystrophic muscle derived from dysferlin-deficient and *mdx*

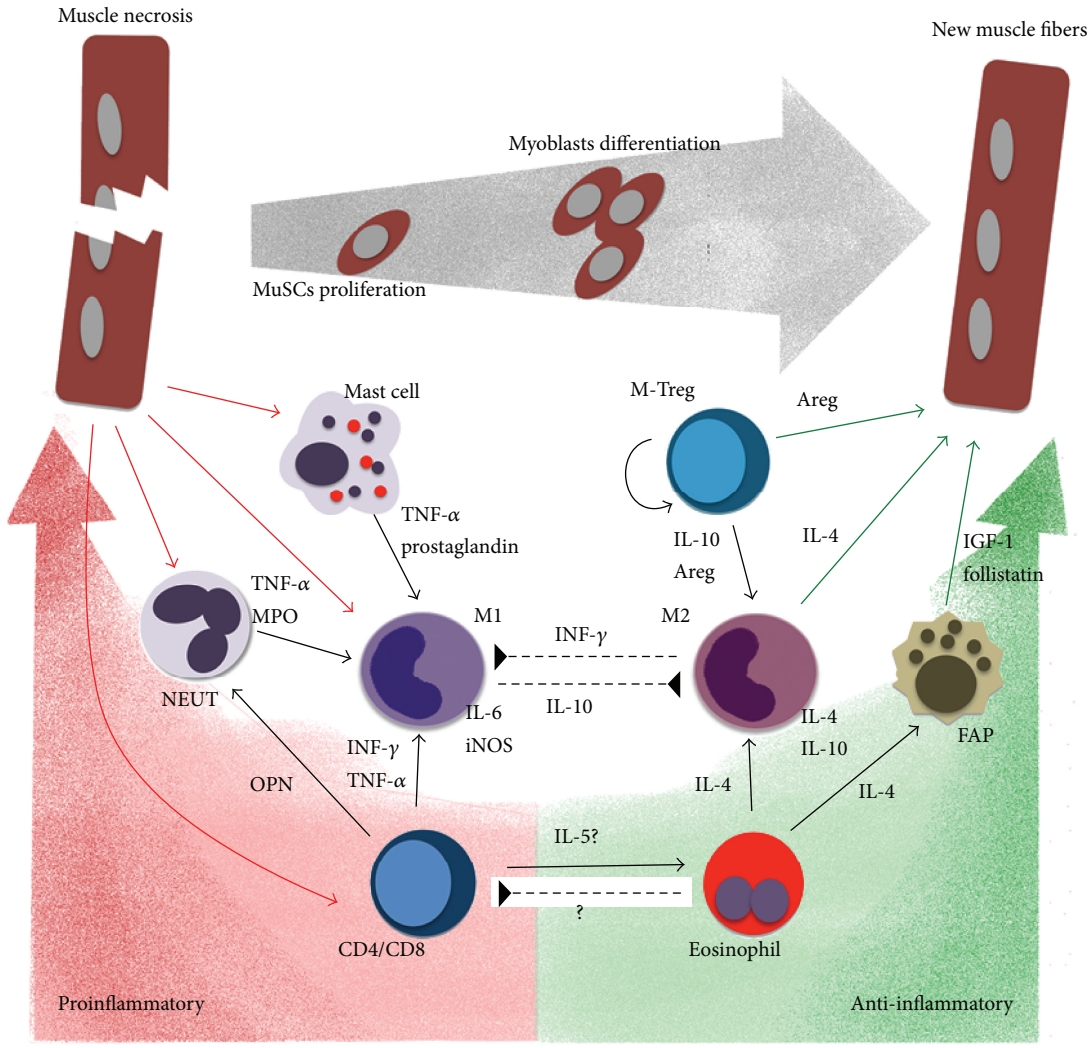


Figure 1: The complexity of the inflammatory milieu in muscle repair. During the initial phases of muscle damage, degenerating fibers release chemokines and cytokines recruiting mast cells, neutrophils (NEUT), and CD8/CD4 T cells which sustain proinflammatory M1 phenotype of recruited monocytes, thus promoting muscle fibers necrosis and debris clearing. These events are primarily mediated by IL-6, NO, TNFα, and IL-1β release. On the other hand, eosinophils are also recruited, which induce FAPs to sustain MuSCs proliferation via IL-4 release. Eosinophils-induced FAPs-produced follistatin promotes MuSCs differentiation, together with the clonally expanding mTreg which produce Areg. Areg also sustains MuSC proliferation and maintains/induces M2 anti-inflammatory/prohealing phenotype, supporting resolution of inflammation. Unbalance in the kinetics, quality, and activity of any of these actors would prevent resolution of inflammation, making muscle environment unfavourable for muscle regeneration, as it occurs in muscular dystrophies.

mice. In particular, in *mdx* muscle, during both the acute and the chronic phase of the disease, this enriched Treg appears to be similar to muscle Treg. Although their number is very low, loss- and gain-of-function experiments showed that reducing Treg cells exacerbates the disease, while enriching them ameliorates it, as measured by serum creatine kinase level, an enzyme released into the blood upon muscle damage and a standard indicator of muscle damage in dystrophic mouse models, as well as by gene expression analysis. Taken together, these findings raise the possibility that a muscle antigen might be involved in recruiting Treg cells to the site of injury and/or retaining them therein. Since endogenous muscle antigens are released in physiological conditions upon injury as well as during pathological conditions by mechanical stress, such as in muscular dystrophies, it is conceivable that Treg cells, which are recruited and/or clonally expanded in muscle following injury, function to prevent a potential systemic autoimmune response by suppressing and regulating the inflammatory response. Taken together, these studies are very important and identify Treg cells and their products as potential new players in the orchestrated series of events underlying muscle repair in both acute and chronic context.

4. Concluding Remarks

Tissue damage induces a series of complex events including inflammation, which culminate in deposition of ECM. If this

process is faulty, excessive and persistent ECM deposition takes place, and normal tissue is substituted by collagen scar, resulting in tissue dysfunction. In muscle, dysregulated repair with persistent fibrosis rather than efficient regeneration plays a prominent role in the clinical decline and reduced life expectancy associated with severe muscular dystrophies. A deep knowledge of the complex events following muscle damage will hopefully allow to design strategies aimed at promoting efficient regeneration.

Although inflammation is now considered a pathological feature of muscle repair, the role and regulation of this process have not been sufficiently examined. Moreover, the relative role of the innate or adaptive immunity in muscle regeneration and dystrophies is still unclear. Although blunting inflammation would not be a "cure" for these diseases, the emerging picture is that multiple strategies, addressing different aspects of the repair process, which may eventually converge, may be successful. In this context, although macrophages are emerging as indispensable for damage control and tissue remodelling following muscle injury, and as principal mediators of pathological skeletal remodelling in diseases such as IIMs and dystrophies, the involvement of other immune cells in promoting or preventing muscle damage resolution is also emerging.

In the last two decades, much research has pointed out the active interactions between muscle cells and the immune system and has clarified some of the mechanisms involved in IIMs. According to most of these studies, muscle cells are now emerging as possible facultative APCs, able, within an inflammatory *milieu,* to drive the activation and proliferation of CD4+ T cells, previously primed against exogenous or endogenous peptides [100]. Whether similar mechanisms may take place in muscle regeneration and muscular dystrophies is not clear yet. It is well known that T cells invade muscle upon both acute injury and chronic diseases. T cells can be recruited by muscle or other infiltrating cell populations, through cytokine release, but their persistence and activity might be sustained by an antigen-specific response. The possibility that an adaptive response to endogenous muscle antigens released upon muscle fibers degeneration, which may represent a common feature in acute injury as well as in IIM or MDs, would attribute to all of these conditions an auto-reactive component. In acute injury, the efficient repair process and the ability of MuSCs to proliferate and differentiate may be sustained by local Treg dependent immune regulation. In fact, autoreactive T cells are normally negatively selected in the thymus; if they escape to the periphery, they are normally eliminated by induction of apoptosis or rendered nonfunctional by the induction of anergy [101]. By contrast, in IIM, muscle fibers are direct targets of adaptive response, and the persistence and clonal expansion of autoreactive T cells prevent muscle healing. Although speculative at this stage, it may be hypothesised that in MDs, the continuous cycles of muscle fiber degeneration, due to muscle fiber fragility, allow for a prolonged and sustained endogenous muscle-specific antigen exposure, leading to the persistence and clonal expansion of potential muscle-antigen reactive T cells, eventually leading to breakdown in peripheral tolerance to self-antigens. Thus, if cytotoxic T cells escape the mechanisms of peripheral tolerance, a cellular immune response will accompany the innate immune response to tissue damage. The presence of alloreactive cytotoxic T cells in *mdx* muscle [102], the ability to transfer pathology from *mdx* mice to healthy mice by adoptive transfer of immune cells primed with muscle homogenates [63], and the presence of a well-conserved peptide in the hypervariable domain of the T-cell receptor of cytotoxic T cells from DMD patients [69] all support the possibility that a breakdown of peripheral tolerance occurs in muscular dystrophy. Furthermore, the observation that muscle specific clonal expansion of Treg cell population occurs during muscle regeneration and in MDs also suggests that a specific adaptive immune response is operating during muscle repair.

The discovery of the possible autoantigens that may evoke an adaptive immune response in MDs will facilitate therapeutic intervention. Similarly, the dissection of the T-cell response, which contributes to the complexity of the inflammatory *milieu* (outlined in Figure 1), will also allow the design of novel therapeutic strategies that would modulate the immune response to create a more favorable environment for MuSCs to differentiate and efficiently regenerate muscle lesions.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors thank Dr. B. Lozanoska-Ochser at DAHFMO, Unit of Histology, for helpful discussion and critical reading of the paper. During the preparation of this review, support was received by the French Muscular Dystrophy Association (AFM) Grant no. 15820 and the Telethon Italy Grant no. GGP13233 to MB. LM was recipient of a Dutch Parent Project NL Fellowship, and, presently, of an AFM Fellowship grant.

References

[1] M. Saclier, S. Cuvellier, M. Magnan, R. Mounier, and B. Chazaud, "Monocyte/macrophage interactions with myogenic precursor cells during skeletal muscle regeneration," *FEBS Journal*, vol. 280, no. 17, pp. 4118–4130, 2013.

[2] A. Mauro, "Satellite cell of skeletal muscle fibers," *The Journal of Biophysical and Biochemical Cytology*, vol. 9, pp. 493–495, 1961.

[3] P. Seale, J. Ishibashi, A. Scimè, and M. A. Rudnicki, "Pax7 is necessary and sufficient for the myogenic specification of CD45+:Sca1+ stem cells from injured muscle," *PLoS Biology*, vol. 2, no. 5, p. E130, 2004.

[4] D. Palacios, C. Mozzetta, S. Consalvi et al., "TNF/p38α/polycomb signaling to Pax7 locus in satellite cells links inflammation to the epigenetic control of muscle regeneration," *Cell Stem Cell*, vol. 7, no. 4, pp. 455–469, 2010.

[5] A. L. Serrano, B. Baeza-Raja, E. Perdiguero, M. Jardí, and P. Muñoz-Cánoves, "Interleukin-6 is an essential regulator

of satellite cell-mediated skeletal muscle hypertrophy," *Cell Metabolism*, vol. 7, no. 1, pp. 33–44, 2008.

[6] S. Kuang, M. A. Gillespie, and M. A. Rudnicki, "Niche regulation of muscle satellite cell self-renewal and differentiation," *Cell Stem Cell*, vol. 2, no. 1, pp. 22–31, 2008.

[7] R. W. Ten Broek, S. Grefte, and J. W. Von Den Hoff, "Regulatory factors and cell populations involved in skeletal muscle regeneration," *Journal of Cellular Physiology*, vol. 224, no. 1, pp. 7–16, 2010.

[8] C. Alexakis, T. Partridge, and G. Bou-Gharios, "Implication of the satellite cell in dystrophic muscle fibrosis: a self-perpetuating mechanism of collagen overproduction," *The American Journal of Physiology—Cell Physiology*, vol. 293, no. 2, pp. C661–C669, 2007.

[9] A. S. Brack, M. J. Conboy, S. Roy et al., "Increased Wnt signaling during aging alters muscle stem cell fate and increases fibrosis," *Science*, vol. 317, no. 5839, pp. 807–810, 2007.

[10] K. A. Lapidos, R. Kakkar, and E. M. McNally, "The dystrophin glycoprotein complex: signaling strength and integrity for the sarcolemma," *Circulation Research*, vol. 94, no. 8, pp. 1023–1031, 2004.

[11] A. Sacco, F. Mourkioti, R. Tran et al., "Short telomeres and stem cell exhaustion model duchenne muscular dystrophy in mdx/mTR mice," *Cell*, vol. 143, no. 7, pp. 1059–1071, 2010.

[12] J. R. Bach and D. Martinez, "Duchenne muscular dystrophy: continuous noninvasive ventilatory support prolongs survival," *Respiratory Care*, vol. 56, no. 6, pp. 744–750, 2011.

[13] A. Crisp, H. Yin, A. Goyenvalle et al., "Diaphragm rescue alone prevents heart dysfunction in dystrophic mice in vitro," *Human Molecular Genetics*, vol. 20, no. 3, pp. 413–421, 2011.

[14] M. Mosqueira, S. M. Baby, S. Lahiri, and T. S. Khurana, "Ventilatory chemosensory drive is blunted in the mdx mouse model of Duchenne Muscular Dystrophy (DMD)," *PLoS ONE*, vol. 8, no. 7, Article ID e69567, 2013.

[15] F. Mourkioti, J. Kustan, P. Kraft et al., "Role of telomere dysfunction in cardiac failure in Duchenne muscular dystrophy," *Nature Cell Biology*, vol. 15, no. 8, pp. 895–904, 2013.

[16] N. P. Evans, S. A. Misyak, J. L. Robertson, J. Bassaganya-Riera, and R. W. Grange, "Dysregulated intracellular signaling and inflammatory gene expression during initial disease onset in duchenne muscular dystrophy," *The American Journal of Physical Medicine and Rehabilitation*, vol. 88, no. 6, pp. 502–522, 2009.

[17] J. E. Heredia, L. Mukundan, F. M. Chen et al., "Type 2 innate signals stimulate fibro/adipogenic progenitors to facilitate muscle regeneration," *Cell*, vol. 153, no. 2, pp. 376–388, 2013.

[18] L. Arnold, A. Henry, F. Poron et al., "Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis," *Journal of Experimental Medicine*, vol. 204, no. 5, pp. 1057–1069, 2007.

[19] B. Deng, M. Wehling-Henricks, S. A. Villalta, Y. Wang, and J. G. Tidball, "IL-10 triggers changes in macrophage phenotype that promote muscle growth and regeneration," *Journal of Immunology*, vol. 189, no. 7, pp. 3669–3680, 2012.

[20] C. Zhang, Y. Li, Y. Wu, L. Wang, X. Wang, and J. Du, "Interleukin-6/signal transducer and activator of transcription 3 (STAT3) pathway is essential for macrophage infiltration and myoblast proliferation during muscle regeneration," *Journal of Biological Chemistry*, vol. 288, no. 3, pp. 1489–1499, 2013.

[21] Y. Kharraz, J. Guerra, C. J. Mann, A. L. Serrano, and P. Muñoz-Cánoves, "Macrophage plasticity and the role of inflammation in skeletal muscle repair," *Mediators of Inflammation*, vol. 2013, Article ID 491497, 9 pages, 2013.

[22] J. G. Tidball and S. A. Villalta, "Regulatory interactions between muscle and the immune system during muscle regeneration," *The American Journal of Physiology—Regulatory Integrative and Comparative Physiology*, vol. 298, no. 5, pp. R1173–R1187, 2010.

[23] P. K. Shireman, V. Contreras-Shannon, O. Ochoa, B. P. Karia, J. E. Michalek, and L. M. McManus, "MCP-1 deficiency causes altered inflammation with impaired skeletal muscle regeneration," *Journal of Leukocyte Biology*, vol. 81, no. 3, pp. 775–785, 2007.

[24] H. X. Nguyen, A. J. Lusis, and J. G. Tidball, "Null mutation of myeloperoxidase in mice prevents mechanical activation of neutrophil lysis of muscle cell membranes in vitro and in vivo," *Journal of Physiology*, vol. 565, no. 2, pp. 403–413, 2005.

[25] S. Hodgetts, H. Radley, M. Davies, and M. D. Grounds, "Reduced necrosis of dystrophic muscle by depletion of host neutrophils, or blocking TNFα function with Etanercept in mdx mice," *Neuromuscular Disorders*, vol. 16, no. 9-10, pp. 591–602, 2006.

[26] H. X. Nguyen and J. G. Tidball, "Null mutation of gp91phox reduces muscle membrane lysis during muscle inflammation in mice," *Journal of Physiology*, vol. 553, no. 3, pp. 833–841, 2003.

[27] J. M. Daley, A. A. Thomay, M. D. Connolly, J. S. Reichner, and J. E. Albina, "Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice," *Journal of Leukocyte Biology*, vol. 83, no. 1, pp. 64–70, 2008.

[28] T. J. Fleming, M. L. Fleming, and T. R. Malek, "Selective expression of Ly-6G on myeloid lineage cells in mouse bone marrow: RB6-8C5 mAb to granulocyte-differentiation antigen (Gr-1) detects members of the Ly-6 family," *Journal of Immunology*, vol. 151, no. 5, pp. 2399–2408, 1993.

[29] N. J. Pillon, P. J. Bilan, L. N. Fink, and A. Klip, "Cross-talk between skeletal muscle and immune cells: muscle-derived mediators and metabolic implications," *The American Journal of Physiology—Endocrinology and Metabolism*, vol. 304, no. 5, pp. E453–E465, 2013.

[30] C. H. Côté, P. Bouchard, N. van Rooijen, D. Marsolais, and E. Duchesne, "Monocyte depletion increases local proliferation of macrophage subsets after skeletal muscle injury," *BMC Musculoskeletal Disorders*, vol. 14, article 359, 2013.

[31] E. Perdiguero, P. Sousa-Victor, V. Ruiz-Bonilla et al., "p38/MKP-1-regulated AKT coordinates macrophage transitions and resolution of inflammation during tissue repair," *Journal of Cell Biology*, vol. 195, no. 2, pp. 307–322, 2011.

[32] T. A. Wynn, A. Chawla, and J. W. Pollard, "Macrophage biology in development, homeostasis and disease," *Nature*, vol. 496, no. 7446, pp. 445–455, 2013.

[33] S. Gordon and P. R. Taylor, "Monocyte and macrophage heterogeneity," *Nature Reviews Immunology*, vol. 5, no. 12, pp. 953–964, 2005.

[34] R. Mounier, M. Théret, L. Arnold et al., "AMPKα1 regulates macrophage skewing at the time of resolution of inflammation during skeletal muscle regeneration," *Cell Metabolism*, vol. 18, no. 2, pp. 251–264, 2013.

[35] S. A. Villalta, H. X. Nguyen, B. Deng, T. Gotoh, and J. G. Tidbal, "Shifts in macrophage phenotypes and macrophage competition for arginine metabolism affect the severity of muscle pathology in muscular dystrophy," *Human Molecular Genetics*, vol. 18, no. 3, pp. 482–496, 2009.

[36] B. Vidal, E. Ardite, M. Suelves et al., "Amelioration of Duchenne muscular dystrophy in mdx mice by elimination of matrix-associated fibrin-driven inflammation coupled to the $\alpha m\beta 2$ leukocyte integrin receptor," *Human Molecular Genetics*, vol. 21, no. 9, pp. 1989–2004, 2012.

[37] M. Wehling, M. J. Spencer, and J. G. Tidball, "A nitric oxide synthase transgene ameliorates muscular dystrophy in mdx mice," *Journal of Cell Biology*, vol. 155, no. 1, pp. 123–131, 2001.

[38] M. Summan, G. L. Warren, R. R. Mercer et al., "Macrophages and skeletal muscle regeneration: a clodronate-containing liposome depletion study," *The American Journal of Physiology—Regulatory Integrative and Comparative Physiology*, vol. 290, no. 6, pp. R1488–R1495, 2006.

[39] C. F. P. Teixeira, S. R. Zamunér, J. P. Zuliani et al., "Neutrophils do not contribute to local tissue damage, but play a key role in skeletal muscle regeneration, in mice injected with Bothrops asper snake venom," *Muscle and Nerve*, vol. 28, no. 4, pp. 449–459, 2003.

[40] J. R. M. Gorospe, B. K. Nishikawa, and E. P. Hoffman, "Recruitment of mast cells to muscle after mild damage," *Journal of the Neurological Sciences*, vol. 135, no. 1, pp. 10–17, 1996.

[41] J. P. Abonia, D. S. Friend, W. G. Austen Jr. et al., "Mast cell protease 5 mediates ischemia-reperfusion injury of mouse skeletal muscle," *Journal of Immunology*, vol. 174, no. 11, pp. 7285–7291, 2005.

[42] H. G. Radley and M. D. Grounds, "Cromolyn administration (to block mast cell degranulation) reduces necrosis of dystrophic muscle in mdx mice," *Neurobiology of Disease*, vol. 23, no. 2, pp. 387–397, 2006.

[43] B. A. Binstadt, P. R. Patel, H. Alencar et al., "Particularities of the vasculature can promote the organ specificity of autoimmune attack," *Nature Immunology*, vol. 7, no. 3, pp. 284–292, 2006.

[44] M. Yokota, K. Suzuki, K. Tokoyoda et al., "Roles of mast cells in the pathogenesis of inflammatory myopathy," *Arthritis Research & Therapy*, vol. 16, no. 2, p. R72, 2014.

[45] M. M. Dimachkie, "Idiopathic inflammatory myopathies," *Journal of Neuroimmunology*, vol. 231, no. 1-2, pp. 32–42, 2011.

[46] L. Casciola-Rosen, K. Nagaraju, P. Plotz et al., "Enhanced autoantigen expression in regenerating muscle cells in idiopathic inflammatory myopathy," *Journal of Experimental Medicine*, vol. 201, no. 4, pp. 591–601, 2005.

[47] D. S. Silberstein and J. R. David, "Tumor necrosis factor enhances eosinophil toxicity to Schistosoma mansoni larvae," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 83, no. 4, pp. 1055–1059, 1986.

[48] N. Ben Baruch-Morgenstern, D. Shik, I. Moshkovits et al., "Paired immunoglobulin-like receptor A is an intrinsic, self-limiting suppressor of IL-5-induced eosinophil development," *Nature Immunology*, vol. 15, no. 1, pp. 36–44, 2014.

[49] Y. Yamaguchi, Y. Hayashi, Y. Sugama et al., "Highly purified murine interleukin 5 (IL-5) stimulates eosinophil function and prolongs in vitro survival. IL-5 as an eosinophil chemotactic factor," *Journal of Experimental Medicine*, vol. 167, no. 5, pp. 1737–1742, 1988.

[50] M. Wehling-henricks, S. Sokolow, J. J. Lee, K. H. Myung, S. A. Villalta, and J. G. Tidball, "Major basic protein-1 promotes fibrosis of dystrophic muscle and attenuates the cellular immune response in muscular dystrophy," *Human Molecular Genetics*, vol. 17, no. 15, pp. 2280–2292, 2008.

[51] B. Cai, M. J. Spencer, G. Nakamura, L. Tseng-Ong, and J. G. Tidball, "Eosinophilia of dystrophin-deficient muscle is promoted by perforin-mediated cytotoxicity by T cell effectors," *The American Journal of Pathology*, vol. 156, no. 5, pp. 1789–1796, 2000.

[52] M. Wehling-Henricks, J. J. Lee, and J. G. Tidball, "Prednisolone decreases cellular adhesion molecules required for inflammatory cell infiltration in dystrophin-deficient skeletal muscle," *Neuromuscular Disorders*, vol. 14, no. 8-9, pp. 483–490, 2004.

[53] V. Horsley, K. M. Jansen, S. T. Mills, and G. K. Pavlath, "IL-4 acts as a myoblast recruitment factor during mammalian muscle growth," *Cell*, vol. 113, no. 4, pp. 483–494, 2003.

[54] A. Uezumi, S.-I. Fukada, N. Yamamoto, S. Takeda, and K. Tsuchida, "Mesenchymal progenitors distinct from satellite cells contribute to ectopic fat cell formation in skeletal muscle," *Nature Cell Biology*, vol. 12, no. 2, pp. 143–152, 2010.

[55] A. W. B. Joe, L. Yi, A. Natarajan et al., "Muscle injury activates resident fibro/adipogenic progenitors that facilitate myogenesis," *Nature Cell Biology*, vol. 12, no. 2, pp. 153–163, 2010.

[56] C. Mozzetta, S. Consalvi, V. Saccone et al., "Fibroadipogenic progenitors mediate the ability of HDAC inhibitors to promote regeneration in dystrophic muscles of young, but not old Mdx mice," *EMBO Molecular Medicine*, vol. 5, no. 4, pp. 626–639, 2013.

[57] N. Cordani, V. Pisa, L. Pozzi, C. Sciorati, and E. Clementi, "Nitric oxide controls fat deposition in dystrophic skeletal muscle by regulating fibro-adipogenic precursor differentiation," *Stem Cells*, vol. 32, no. 4, pp. 874–885, 2014.

[58] J.-H. Choi, Y.-E. Park, S.-I. Kim et al., "Differential Immunohistological features of inflammatory myopathies and dysferlinopathy," *Journal of Korean Medical Science*, vol. 24, no. 6, pp. 1015–1023, 2009.

[59] M. Marino, F. Scuderi, P. Mazzarelli, F. Mannella, C. Provenzano, and E. Bartoccioni, "Constitutive and cytokine-induced expression of MHC and intercellular adhesion molecule-1 (ICAM-1) on human myoblasts," *Journal of Neuroimmunology*, vol. 116, no. 1, pp. 94–101, 2001.

[60] J. Schmidt, G. Rakocevic, R. Raju, and M. C. Dalakas, "Upregulated inducible co-stimulator (ICOS) and ICOS-ligand in inclusion body myositis muscle: significance for CD8+ T cell cytotoxicity," *Brain*, vol. 127, no. 5, pp. 1182–1190, 2004.

[61] A. Lerario, F. Cogiamanian, C. Marchesi et al., "Effects of rituximab in two patients with dysferlin-deficient muscular dystrophy," *BMC Musculoskeletal Disorders*, vol. 11, article 157, 2010.

[62] M. Salajegheh, J. L. Pinkus, A. A. Amato et al., "Permissive environment for B-cell maturation in myositis muscle in the absence of B-cell follicles," *Muscle and Nerve*, vol. 42, no. 4, pp. 576–583, 2010.

[63] M. J. Spencer, E. Montecino-Rodriguez, K. Dorshkind, and J. G. Tidball, "Helper ($CD4^+$) and cytotoxic ($CD8^+$) T cells promote the pathology of dystrophin-deficient muscle," *Clinical Immunology*, vol. 98, no. 2, pp. 235–243, 2001.

[64] S. Eghtesad, S. Jhunjhunwala, S. R. Little, and P. R. Clemens, "Rapamycin ameliorates dystrophic phenotype in mdx mouse skeletal muscle," *Molecular Medicine*, vol. 17, no. 9-10, pp. 917–924, 2011.

[65] C. D. Tsoukas, B. Landgraf, J. Bentin et al., "Activation of resting T lymphocytes by anti-CD3 (T3) antibodies in the absence of monocytes," *Journal of Immunology*, vol. 135, no. 3, pp. 1719–1723, 1985.

[66] N. Al-Shanti, P. Durcan, S. Al-Dabbagh, G. A. Dimchev, and C. E. Stewart, "Activated lymphocytes secretome inhibits

differentiation and induces proliferation of C2C12 myoblasts," *Cellular Physiology and Biochemistry*, vol. 33, no. 1, pp. 117–128, 2014.

[67] J. T. Kissel, D. J. Lynn, K. W. Rammohan et al., "Mononuclear cell analysis of muscle biopsies in prednisone- and azathioprine-treated Duchenne muscular dystrophy," *Neurology*, vol. 43, no. 3, pp. 532–536, 1993.

[68] J. R. Mendell, R. T. Moxley, R. C. Griggs et al., "Randomized, double-blind six-month trial of prednisone in Duchenne's muscular dystrophy," *The New England Journal of Medicine*, vol. 320, no. 24, pp. 1592–1597, 1989.

[69] E. Gussoni, G. K. Pavlath, R. G. Miller et al., "Specific T cell receptor gene rearrangements at the site of muscle degeneration in Duchenne muscular dystrophy," *Journal of Immunology*, vol. 153, no. 10, pp. 4798–4805, 1994.

[70] R. Mantegazza, F. Andreetta, P. Bernasconi et al., "Analysis of T cell receptor repertoire of muscle-infiltrating T lymphocytes in polymyositis. Restricted Vα/β rearrangements may indicate antigen-driven selection," *Journal of Clinical Investigation*, vol. 91, no. 6, pp. 2880–2886, 1993.

[71] S. Gordon, "Alternative activation of macrophages," *Nature Reviews Immunology*, vol. 3, no. 1, pp. 23–35, 2003.

[72] D. Burzyn, W. Kuswanto, D. Kolodin et al., "A special population of regulatory T cells potentiates muscle repair," *Cell*, vol. 155, no. 6, pp. 1282–1295, 2013.

[73] P. Wang and S. G. Zheng, "Regulatory T cells and B cells: implication on autoimmune diseases," *International Journal of Clinical and Experimental Pathology*, vol. 6, no. 12, pp. 2668–2674, 2013.

[74] A. Farini, M. Meregalli, M. Belicchi et al., "T and B lymphocyte depletion has a marked effect on the fibrosis of dystrophic skeletal muscles in the scid/mdx mouse," *Journal of Pathology*, vol. 213, no. 2, pp. 229–238, 2007.

[75] J. Morrison, D. B. Palmer, S. Cobbold, T. Partridge, and G. Bou-Gharios, "Effects of T-lymphocyte depletion on muscle fibrosis in the mdx mouse," *The American Journal of Pathology*, vol. 166, no. 6, pp. 1701–1710, 2005.

[76] D. Vallese, E. Negroni, S. Duguez et al., "The Rag2^{-}Il2rb^{-}Dmd^{-} mouse: a novel dystrophic and immunodeficient model to assess innovating therapeutic strategies for muscular dystrophies," *Molecular Therapy*, vol. 21, no. 10, pp. 1950–1957, 2013.

[77] S. A. Vetrone, E. Montecino-Rodriguez, E. Kudryashova et al., "Osteopontin promotes fibrosis in dystrophic mouse muscle by modulating immune cell subsets and intramuscular TGF-β," *Journal of Clinical Investigation*, vol. 119, no. 6, pp. 1583–1594, 2009.

[78] L. Madaro, A. Pelle, C. Nicoletti et al., "PKC theta ablation improves healing in a mouse model of muscular dystrophy," *PLoS ONE*, vol. 7, no. 2, Article ID e31515, 2012.

[79] Z. Gao, Z. Wang, X. Zhang et al., "Inactivation of PKCθ leads to increased susceptibility to obesity and dietary insulin resistance in mice," *The American Journal of Physiology—Endocrinology and Metabolism*, vol. 292, no. 1, pp. E84–E91, 2007.

[80] L. Madaro, V. Marrocco, P. Fiore et al., "PKCθ signaling is required for myoblast fusion by regulating the expression of caveolin-3 and β1D integrin upstream focal adhesion kinase," *Molecular Biology of the Cell*, vol. 22, no. 8, pp. 1409–1419, 2011.

[81] G. Messina, S. Biressi, S. Monteverde et al., "Nfix regulates fetal-specific transcription in developing skeletal muscle," *Cell*, vol. 140, no. 4, pp. 554–566, 2010.

[82] M. D'Andrea, A. Pisaniello, C. Serra et al., "Protein kinase C theta co-operates with calcineurin in the activation of slow muscle genes in cultured myogenic cells," *Journal of Cellular Physiology*, vol. 207, no. 2, pp. 379–388, 2006.

[83] L. Madaro, V. Marrocco, S. Carnio, M. Sandri, and M. Bouché, "Intracellular signaling in ER stress-induced autophagy in skeletal muscle cells," *The FASEB Journal*, vol. 27, no. 5, pp. 1990–2000, 2013.

[84] J. O. Valenzuela, C. Iclozan, M. S. Hossain et al., "PKCθ is required for alloreactivity and GVHD but not for immune responses toward leukemia and infection in mice," *Journal of Clinical Investigation*, vol. 119, no. 12, pp. 3774–3786, 2009.

[85] T. Gruber, N. Hermann-Kleiter, C. Pfeifhofer-Obermair et al., "PKCθ cooperates with PKCα in alloimmune responses of T cells in vivo," *Molecular Immunology*, vol. 46, no. 10, pp. 2071–2079, 2009.

[86] B. J. Marsland, T. J. Soos, G. Späth, D. R. Littman, and M. Kopf, "Protein kinase C θ is critical for the development of in vivo T helper (Th)2 cell but not Th-1 cell responses," *Journal of Experimental Medicine*, vol. 200, no. 2, pp. 181–189, 2004.

[87] A. M. Healy, E. Izmailova, M. Fitzgerald et al., "PKC-θ-deficient mice are protected from th1-dependent antigen-induced arthritis," *Journal of Immunology*, vol. 177, no. 3, pp. 1886–1893, 2006.

[88] S.-L. Tan, J. Zhao, C. Bi et al., "Resistance to experimental autoimmune encephalomyelitis and impaired IL-17 production in protein kinase Cθ-deficient mice," *Journal of Immunology*, vol. 176, no. 5, pp. 2872–2879, 2006.

[89] K. Nagahama, A. Ogawa, K. Shirane, Y. Shimomura, K. Sugimoto, and A. Mizoguchi, "Protein kinase C theta plays a fundamental role in different types of chronic colitis," *Gastroenterology*, vol. 134, no. 2, pp. 459–469, 2008.

[90] A. Zanin-Zhorov, Y. Ding, S. Kumari et al., "Protein kinase C-θ mediates negative feedback on regulatory T cell function," *Science*, vol. 328, no. 5976, pp. 372–376, 2010.

[91] J. Ma, Y. Ding, X. Fang, R. Wang, and Z. Sun, "Protein kinase C-θ inhibits inducible regulatory T cell differentiation via an AKT-Foxo1/3a-dependent pathway," *Journal of Immunology*, vol. 188, no. 11, pp. 5337–5347, 2012.

[92] A. Farini, C. Sitzia, C. Navarro et al., "Absence of T and B lymphocytes modulates dystrophic features in dysferlin deficient animal model," *Experimental Cell Research*, vol. 318, no. 10, pp. 1160–1174, 2012.

[93] R. Han and K. P. Campbell, "Dysferlin and muscle membrane repair," *Current Opinion in Cell Biology*, vol. 19, no. 4, pp. 409–416, 2007.

[94] H. Liao, E. Franck, M. Fréret et al., "Myoinjury transiently activates muscle antigen-specific CD8+ T cells in lymph nodes in a mouse model," *Arthritis and Rheumatism*, vol. 64, no. 10, pp. 3441–3451, 2012.

[95] N. A. Young, R. Sharma, A. K. Friedman, B. H. Kaffenberger, B. Bolon, and W. N. Jarjour, "Aberrant muscle antigen exposure in mice is sufficient to cause myositis in a Treg cell-deficient milieu," *Arthritis & Rheumatism*, vol. 65, no. 12, pp. 3259–3270, 2013.

[96] S. Chakrabarti, K. S. Kobayashi, R. A. Flavell et al., "Impaired membrane resealing and autoimmune myositis in synaptotagmin VII-deficient mice," *Journal of Cell Biology*, vol. 162, no. 4, pp. 543–549, 2003.

[97] S. Z. Josefowicz, "Regulators of chromatin state and transcription in CD4 T-cell polarization," *Immunology*, vol. 139, no. 3, pp. 299–308, 2013.

[98] J. P. Golding, E. Calderbank, T. A. Partridge, and J. R. Beauchamp, "Skeletal muscle stem cells express anti-apoptotic ErbB receptors during activation from quiescence," *Experimental Cell Research*, vol. 313, no. 2, pp. 341–356, 2007.

[99] E. R. Andrechek, W. R. Hardy, A. A. Girgis-Gabardo et al., "ErbB2 is required for muscle spindle and myoblast cell survival," *Molecular and Cellular Biology*, vol. 22, no. 13, pp. 4714–4722, 2002.

[100] H. Wiendl, R. Hohlfeld, and B. C. Kieseier, "Immunobiology of muscle: advances in understanding an immunological microenvironment," *Trends in Immunology*, vol. 26, no. 7, pp. 373–380, 2005.

[101] M. Srinivasan and K. A. Frauwirth, "Peripheral tolerance in CD8+ T cells," *Cytokine*, vol. 46, no. 2, pp. 147–159, 2009.

[102] M. J. Spencer, C. M. Walsh, K. A. Dorshkind, E. M. Rodriguez, and J. G. Tidball, "Myonuclear apoptosis in dystrophic mdx muscle occurs by perforin-mediated cytotoxicity," *Journal of Clinical Investigation*, vol. 99, no. 11, pp. 2745–2751, 1997.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 124063, 15 pages
http://dx.doi.org/10.1155/2014/124063

Research Article

Cardioprotective Effects of Osteopontin-1 during Development of Murine Ischemic Cardiomyopathy

Georg D. Duerr,[1] Bettina Mesenholl,[1,2] Jan C. Heinemann,[1] Martin Zoerlein,[1] Peter Huebener,[1,3] Prisca Schneider,[1] Andreas Feisst,[1,4] Alexander Ghanem,[5] Klaus Tiemann,[5,6] Daniela Dewald,[7] Armin Welz,[1] and Oliver Dewald[1]

[1] *Department of Cardiac Surgery, University Clinical Centre Bonn, Sigmund-Freud-Straße 25, 53105 Bonn, Germany*
[2] *Department of Pediatric Cardiology, University Clinical Centre Bonn, Sigmund-Freud-Straße 25, 53105 Bonn, Germany*
[3] *Department of Medicine I, University Clinical Center Hamburg-Eppendorf, Martinistr. 52, 20251 Hamburg, Germany*
[4] *Department of Radiology, University Clinical Centre Bonn, Sigmund-Freud-Straße 25, 53105 Bonn, Germany*
[5] *Department of Medicine II and Cardiology, University Clinical Centre Bonn, Sigmund-Freud-Straße 25, 53105 Bonn, Germany*
[6] *Isar Heart Center, Isar Kliniken, Sonnenstraße 24-26, 80331 Munich, Germany*
[7] *Department of Anesthesiology and Intensive Care, University Clinical Centre Bonn, Sigmund-Freud-Straße 25, 53105 Bonn, Germany*

Correspondence should be addressed to Oliver Dewald; o.dewald@uni-bonn.de

Received 27 January 2014; Revised 21 April 2014; Accepted 23 April 2014; Published 29 May 2014

Academic Editor: Dario Coletti

Repetitive brief ischemia and reperfusion (I/R) is associated with ventricular dysfunction in pathogenesis of murine ischemic cardiomyopathy and human hibernating myocardium. We investigated the role of matricellular protein osteopontin-1 (OPN) in murine model of repetitive I/R. One 15-min LAD-occlusion followed by reperfusion was performed daily over 3, 5, and 7 consecutive days in C57/Bl6 wildtype- (WT-) and $OPN^{-/-}$-mice ($n = 8$/group). After echocardiography hearts were processed for histological and mRNA-studies. Cardiac fibroblasts were isolated, cultured, and stimulated with TGF-β1. WT-mice showed an early, strong, and cardiomyocyte-specific osteopontin-expression leading to interstitial macrophage infiltration and consecutive fibrosis after 7 days I/R in absence of myocardial infarction. In contrast, $OPN^{-/-}$-mice showed small, nontransmural infarctions after 3 days I/R associated with significantly worse ventricular dysfunction. $OPN^{-/-}$-mice had different expression of myocardial contractile elements and antioxidative mediators and a lower expression of chemokines during I/R. $OPN^{-/-}$-mice showed predominant collagen deposition in macrophage-rich small infarctions. We found lower induction of tenascin-C, MMP-9, MMP-12, and TIMP-1, whereas MMP-13-expression was higher in $OPN^{-/-}$-mice. Cultured $OPN^{-/-}$-myofibroblasts confirmed these findings. In conclusion, osteopontin seems to modulate expression of contractile elements, antioxidative mediators, and inflammatory response and subsequently remodel in order to protect cardiomyocytes in murine ischemic cardiomyopathy.

1. Introduction

Clinical and experimental studies provided evidence to establish the concept of repetitive episodes of brief ischemia and reperfusion (I/R) as an important mechanism in the development of ischemic heart disease [1, 2]. Repetitive I/R leads to progressive morphological and functional changes in human heart resulting in hibernating myocardium. Hibernating myocardium is a condition, where myocardial function is reduced under poor substrate availability in order to prevent cardiomyocyte loss. The hallmark of this condition is the reversibility of ventricular dysfunction upon restoration of coronary blood flow—reperfusion. The presence of newly recruited inflammatory cells in human hibernating myocardium has been shown to be beneficial for the restoration of left ventricular function after revascularization [3]. In order to better understand the underlying mechanisms, we developed a murine model of repetitive, brief I/R [4]. This model shares pathological and functional features of the human hibernating myocardium including a transient inflammatory reaction, interstitial fibrosis, and reversible left ventricular dysfunction. We described an important role for

the chemokine CCL2 in development of interstitial fibrosis and impaired ventricular function in this model [5] and also found a strong induction of osteopontin during I/R.

Osteopontin is a matricellular protein and cytokine involved in the regulation of macrophage function and as a remodeling-associated mediator in different tissues [6]. Expression of osteopontin was associated with important fibrogenic signals during development of *bleomycin*-induced lung fibrosis [7]. Osteopontin expression was required for differentiation of myofibroblasts *in vitro* [8]. Osteopontin has also been associated with development of fibrosis and cardiac adaptation in angiotensin II-induced murine myocardial hypertrophy [9]. Mice lacking osteopontin revealed exaggerated left ventricular dilation and reduced collagen deposition after nonreperfused myocardial infarction [10]. On the other hand, cardiac-specific overexpression of osteopontin led to the development of dilated cardiomyopathy being lethal in mice at 12 weeks of age [11]. In a recent study, osteopontin has further been suggested to have a major influence on myocardial remodeling in desmin-deficient mice, which suffer from myocardial degeneration without any kind of injury [12]. In contrast to the published data, we investigated the role of osteopontin in development of ischemic cardiomyopathy using our model of repetitive, brief I/R without myocardial infarction.

We describe novel aspects of osteopontin-dependent cardioprotection in murine ischemic myocardium influencing expression of contractile elements and chemokines, as well as regulation of remodeling factors tenascin-C and matrix metalloproteinases (MMP).

2. Material and Methods

2.1. Study Animals. All mouse experiments were performed on 20–25 g and 10–16-week-old mice. We used wild type- (WT-) C57BL/6J mice (Charles River, Sulzfeld, Germany) and the homozygote osteopontin-1-deficient- ($OPN^{-}/^{-}$-) mice, which were derived on C57BL/6J-background and provided by Rittling [13]. All experiments were performed in accordance with an animal protocol approved by the local governmental authorities and according to the EU Directive 2010/63/EU for animal experiments.

2.2. Mouse Model of Repetitive I/R. Briefly, in an initial surgery, an 8-0 Prolene suture (Ethicon, Norderstedt, Germany) was placed around the left descending coronary artery and stored subcutaneously as previously described [4]. After a recovery period of 7 days following initial surgery trauma, repetitive I/R was performed. One daily episode of 15 min coronary artery occlusion was followed by reperfusion until the next day. This protocol was repeated over 1, 3, 5, or 7 consecutive days. Left ventricular function was assessed via echocardiography after 7 days I/R before heart retrieval for histology group. Hearts were excised and fixated in zinc-paraformaldehyde (Z-fix, 4%; Anatech, Battle Creek, MI, USA) for histology, stored in RNAlater (Qiagen, Hilden, Germany) for mRNA-studies, or dissolved for cardiomyocyte isolation.

2.3. Echocardiography. Mice were anesthetized with isoflurane under continuous heart rate monitoring to minimize cardiodepressive effects as previously described [4]. We used a commercially available ultrasound system (HDI-5000; ATL-Phillips, Oceanside, CA, USA) equipped with a linear array transducer (CL15-7) operating at 15 MHz and providing frame rates up to 284 Hz. Briefly, 2D-guided M-mode data was acquired in the parasternal short-axis view at the level of the papillary muscle. Fractional shortening as a global left ventricular function parameter and anterior wall thickening as a regional parameter of left ventricular function were calculated as previously described [4].

2.4. Histology and Immunohistochemistry. Basic histological evaluation was performed via haematoxylin and eosin staining. Picrosirius red staining was used to visualize collagen deposition and quantitative planimetric analysis of collagen stained area was performed as already published [4]. Immunohistochemical staining was performed using Vectastain Elite ABC kits and diaminobenzidine (AXXORA, Lörrach, Germany). We used the following antibodies: α-smooth muscle actin (α-smac; Sigma, Steinheim, Germany), monocyte-specific rat anti-mouse antibody F4/80 (Abcam, Cambridge, UK), tenascin-C rabbit anti-chicken polyclonal antibody (Chemicon, Temecula, CA, USA) *in vivo*, and troponin T (Fisher Scientific, Schwerte, Germany) and CD31 (BD-Pharmingen, Heidelberg, Germany) *in vitro*. F4/80-positive cells were counted manually in order to ascertain their monocyte/macrophage morphology.

2.5. Cardiomyocyte Isolation. We extracted cardiomyocytes from hearts after 3 days I/R using Langendorff-apparatus (Radnoti Technologies Inc., Monrovia, CA, USA) in order to measure the cardiomyocyte specific mRNA-expression of osteopontin as previously described [14]. Briefly, ischemic hearts were perfused with collagenase B (Roche, Mannheim, Germany) for digestion. The eluate containing the cardiomyocytes was subjected to manual cell count immediately after isolation. Cell count of troponin T stained cells revealed >90% of cells being cardiomyocytes. The isolated cells were transferred to Trizol (Invitrogen, Karlsruhe, Germany) for subsequent mRNA-isolation. The whole isolation protocol was performed within 20 min.

2.6. Cardiac Fibroblast Cell Culture. Fibroblasts were isolated from nonischemic mouse hearts according to a slightly modified protocol [15]. Briefly, three noninfarcted, naïve WT- or $OPN^{-}/^{-}$-hearts were pooled for one sample. Each group consisted of $n = 5$–7 pooled samples for further experiments. The hearts were dissected free of vessels and atria, transferred to 1 mL of collagenase buffer (Roche), and quickly minced into small pieces with scalpel and microforceps. This digestion process continued until no visible tissue fragments were left. Next, the cell suspensions of 3 mice were pelleted, washed, combined, and plated on a T75 tissue-culture flask (Invitrogen) in full medium supplemented with 10% of FBS (Invitrogen) and antibiotic/antimycotic solution (Invitrogen). After 24 h incubation, nonadherent cells were removed by discarding the supernatant. Upon reaching

confluence, cells were detached with trypsin/EDTA (Invitrogen), split in a 1 : 2 or 1 : 4 ratio, and recultured. Characteristic fibroblast morphology was determined visually under a light microscope. Only fibroblasts at passages 2–4 were used for experiments. Pure fibroblast cultures were confirmed by immunocytochemistry using antibodies against α-smac, troponin T, F4/80, and CD31. Cells were stimulated with human TGF-β1 (100 ng/mL; PeproTech, Hamburg, Germany) and cultured under normoxia (21% O_2, 5% CO_2, 37°C) or hypoxic conditions (2% O_2, 5% CO_2, 37°C) for 6 and 24 h. At the end of the experiment, cells were harvested and stored in Trizol (Invitrogen) at −80°C until RNA extraction.

2.7. RNA-Extraction and Taqman RT-qPCR. The mRNA-expression was determined using Taqman real time quantitative PCR system (RT-qPCR, Applied Biosystems, Foster City, CA, USA). Total RNA was isolated with a standard protocol using phenole/chloroform extraction (Trizol, Invitrogen). First-strand cDNA was synthesized using the high capacity cDNA transcription kit (Applied Biosystems) with random hexameric primers as described in the manufacturer's protocol. cDNA was diluted 1/10 and analyzed in RT-qPCR according to the manufacturer's instructions on an ABI Prism 7900 HT Sequence Detection System using SDS2.2 Software (Applied Biosystems). Target gene-expression was normalized to an internal control and to GAPDH for *in vivo* experiments or 18 S for *in vitro* studies. All primers were measured using FAM-TAMRA chemistry and the relative standard curve method. Dissociation curve analysis was performed to ascertain the amplification of a single PCR product.

2.8. Statistical Analysis. Data were normally distributed and presented as mean ± S.E.M. Two way ANOVA with a Student's Newman-Keuls' corrected post hoc analysis was done to compare differences between the groups (Prism 5.0; Graph Pad, La Jolla, CA, USA). Differences with $P \leq 0.05$ were considered significant.

3. Results

3.1. OPN^{-}/$^{-}$-Mice Develop Small, Nontransmural Infarctions during Repetitive I/R Episodes. Brief repetitive I/R led to ~350-fold induction of osteopontin in WT-mice after 3 and 5 days of the ischemic protocol (Figure 1(a)). In order to investigate whether osteopontin was produced by cardiomyocytes in ischemic heart, we isolated cardiomyocytes after 3 days I/R from WT-hearts via Langendorff-perfusion and found a significant 9-fold increase in osteopontin mRNA-expression (Figure 1(b)). Naïve and sham operated OPN^{-}/$^{-}$-mice presented with normal myocardial morphology and cardiac function when compared with WT-mice, as previously described [10]. Repetitive I/R caused no loss of cardiomyocytes after 7 days I/R in WT-mice (Figure 1(c)), as previously reported [4]. In contrast, after 3 days I/R OPN^{-}/$^{-}$-mice showed an irreversible loss of cardiomyocytes in small areas of nontransmural infarctions and a mostly consolidated scar formation after 7 days I/R in these regions (Figure 1(d)). These small, nontransmural infarctions were scattered throughout the ischemic area of the left ventricular anterior wall. Echocardiography measurement of fractional shortening revealed significant dysfunction in both strains when compared to their shams (Figure 1(e)). The left ventricular dysfunction was also significantly worse in OPN^{-}/$^{-}$-mice compared to the WT-mice. Anterior wall thickening measurement showed comparable degree of regional dysfunction in ischemic anterior wall between both strains after 7 days I/R, but only ischemic OPN^{-}/$^{-}$-hearts demonstrated with significantly worse left ventricular function compared to their shams (Figure 1(f)).

3.2. Ischemic OPN^{-}/$^{-}$-Hearts Show Different Expression of MHC- and Actin-Isoforms and Antioxidative Mediators. In order to investigate the underlying mechanisms for the irreversible loss of cardiomyocytes in OPN^{-}/$^{-}$-hearts, we measured myosin heavy chain (MHC) isoforms. OPN^{-}/$^{-}$-mice were unable to decrease expression of more ATP consuming and therefore energetically unfavorable α-MHC in contrast to WT-mice during repetitive I/R (Figure 2(a)). At the same time, OPN^{-}/$^{-}$-mice showed up to 5-fold higher expression of less ATP demanding β-MHC after 7 days I/R than WT-mice (Figure 2(b)). Despite of this adaptation, continuous expression of α-MHC might result in unfavorable utilization of ATP in ischemic osteopontin-deficient myocardium. We further investigated the contractile elements and measured mRNA-expression of skeletal and cardiac actin isoforms. While WT-hearts showed practically unchanged expression of skeletal actin during repetitive I/R, while their OPN^{-}/$^{-}$-mice counterparts experienced a significant ~4-fold upregulation after 5 days I/R, when compared to respective shams or WT-group (Figure 2(c)). The expression of cardiac specific actin was downregulated in WT-hearts to as low as 14% of sham expression level during repetitive I/R protocol (Figure 2(d)). Interestingly, OPN^{-}/$^{-}$-hearts showed a continuous and significant upregulation of cardiac actin when compared to the respective WT-groups. As an additional marker of cardiac remodeling, we measured mRNA expression of desmin and found no induction of it in WT-hearts, in contrast to a significantly higher expression of it (max. 2.5-fold) in OPN^{-}/$^{-}$-hearts (Figure 2(e)).

OPN^{-}/$^{-}$-mice showed a significant 3-fold lower mRNA-induction of antioxidative mediator heme oxygenase-1 after 3 days of I/R and a tendency towards less induction thereafter (Figure 3(a)). OPN^{-}/$^{-}$-mice also presented with a tendency to less induction of glutathione peroxidase-1 during I/R (Figure 3(b)). Furthermore, their expression of zinc-storage and antioxidative proteins metallothionein-1 and -2 was significantly decreased during the I/R-protocol (Figures 3(c) and 3(d)). These differences in expression of contractile elements and induction of antioxidative mediators seem to be the major factors responsible for the irreversible damage of the cardiomyocytes in remote perfusion areas, which histologically presented as small, nontransmural infarcted areas.

3.3. Differences in Cellular and Molecular Response during Postischemic Inflammation. In the next step we investigated inflammatory reaction and found a different pattern of macrophage distribution between the genotypes. Macrophages were evenly distributed in the interstitial

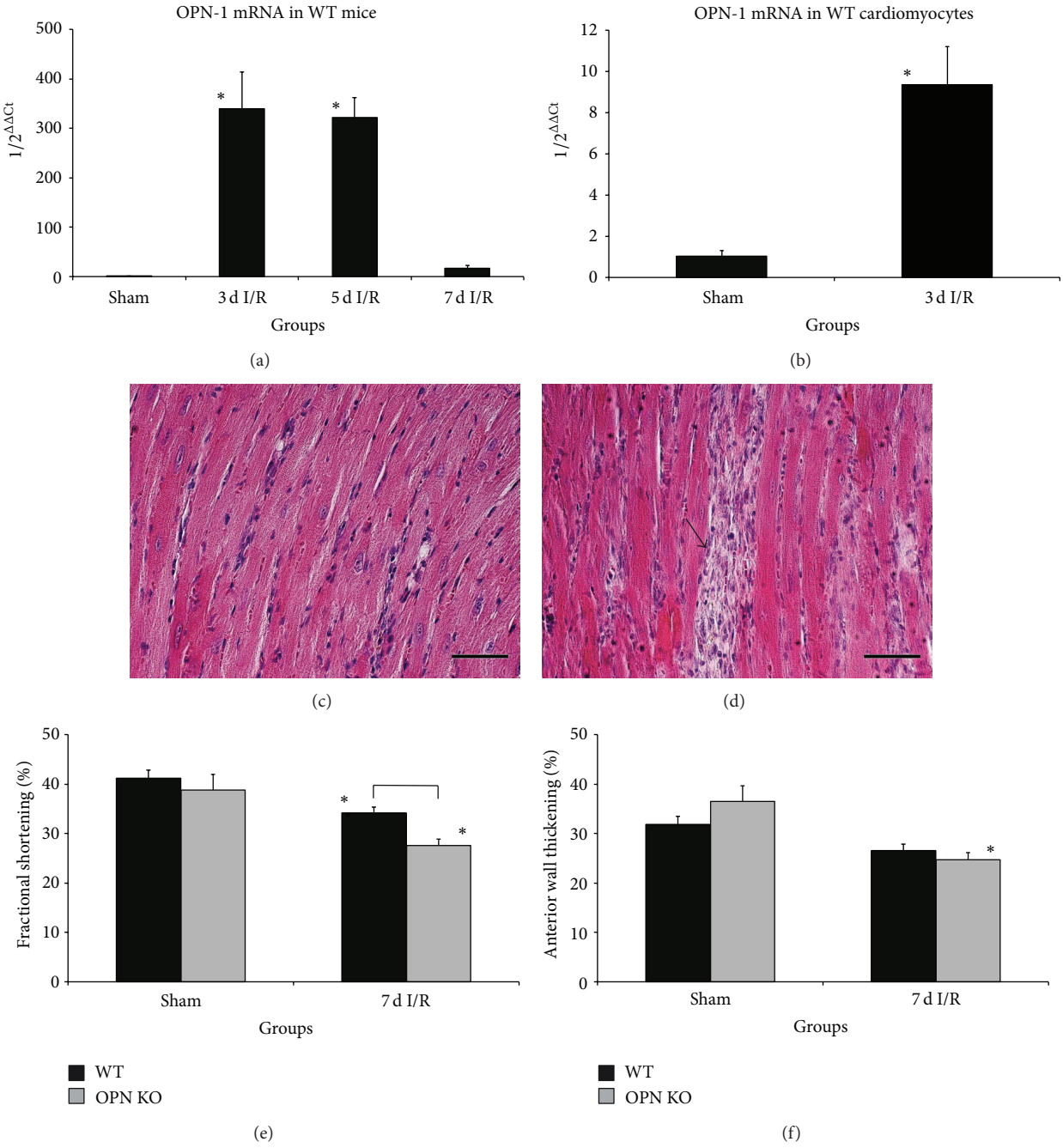


Figure 1: Osteopontin-deficiency leads to cardiomyocyte loss and poor left ventricular function. (a) Osteopontin mRNA-expression in whole WT-mouse hearts during repetitive I/R and (b) in Langendorff-isolated adult WT-cardiomyocytes after 3 days I/R compared to sham. (c) Representative HE-stained section of WT-heart after 7 days I/R shows interstitial cellular infiltration in contrast to (d) irreversible loss of cardiomyocytes in OPN^{-}/$^{-}$-mice (arrow). (e) Fractional shortening was impaired in both strains, but loss of left ventricular contractility was significantly reduced in OPN^{-}/$^{-}$-mice after 7 d I/R compared to WT. (f) Anterior wall thickening was significantly reduced in OPN^{-}/$^{-}$-mice after 7 days I/R. n = 8–11/group. Scale bars represent 50 μm. RT-qPCR using Taqman and mRNA-expression is related to controls and GAPDH using comparative $\Delta\Delta$Ct-method. Bracket indicates $P \leq 0.05$ between genotypes; $*$ indicates $P \leq 0.05$ versus respective shams.

space of ischemic WT-hearts after 7 days I/R (Figure 4(a)). In contrast, the OPN^{-}/$^{-}$-mice presented with increased macrophage infiltration into small, nontransmural infarctions (Figure 4(b)). The quantitative analysis of F4/80 staining revealed a comparable macrophage density after 3 and 7 days I/R in both genotypes (Figure 4(c)). Still, at the maximum of macrophage action after 5 days I/R we found a significant 46% higher cell density in OPN^{-}/$^{-}$-hearts than in WT-hearts. Further analysis of cellular localization showed a significantly stronger (2-3-fold) macrophage infiltration of the interstitial space in WT-hearts than in the very rarely observed small infarcted areas (Figure 4(d)). At the same

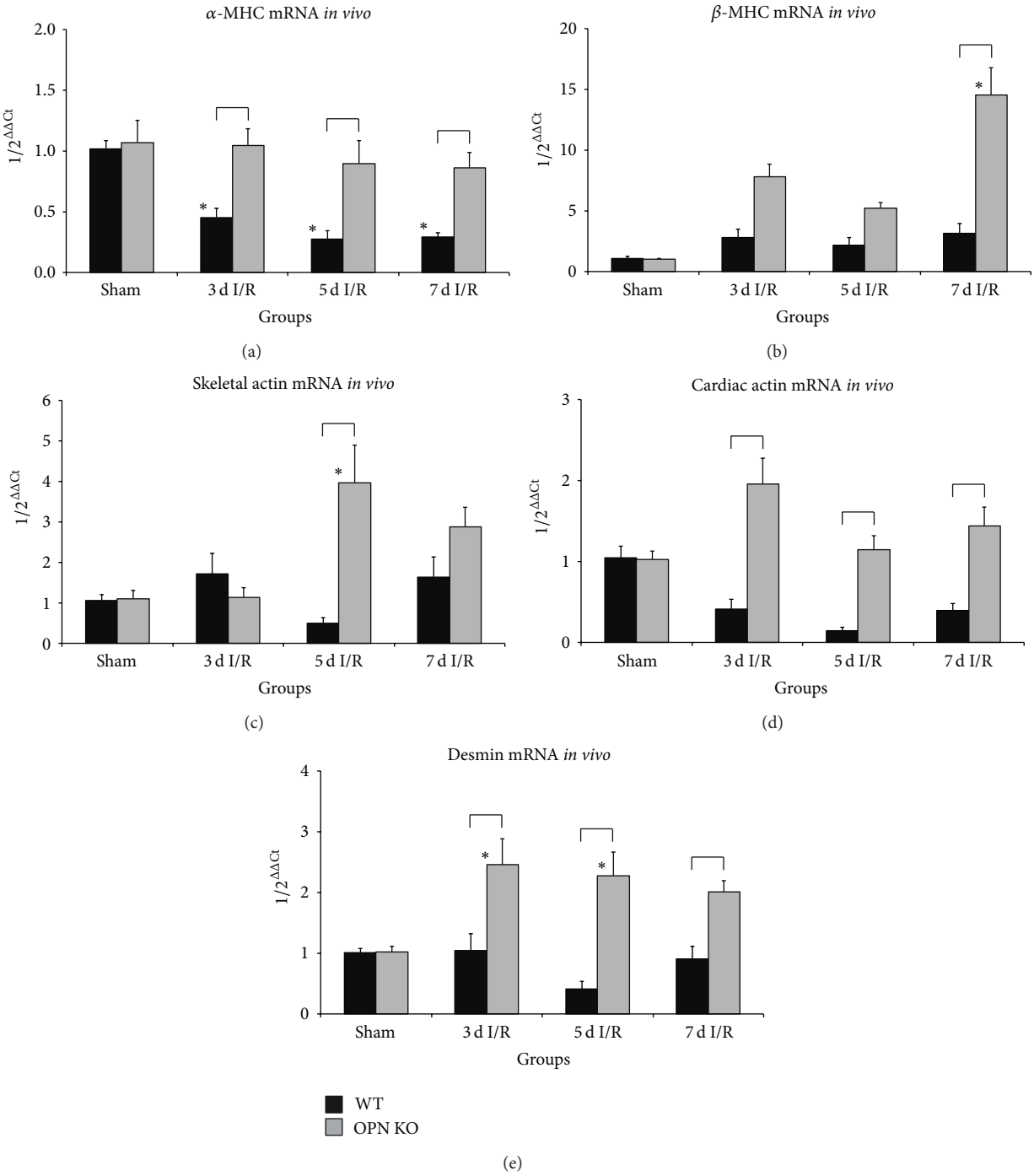


Figure 2: Ischemic OPN$^{-/-}$-hearts show different expression contractile elements. mRNA-expression of (a) α-myosin heavy chain (α-MHC), (b) β-MHC, (c) skeletal actin, (d) cardiac actin, and (e) desmin in OPN$^{-/-}$-mice compared to WT-mice during repetitive I/R. n = 8-9/group. RT-qPCR using Taqman and mRNA-expression is related to controls and GAPDH using comparative ΔΔCt-method. Bracket indicates $P \leq 0.05$ between genotypes; $*$ indicates $P \leq 0.05$ versus respective shams.

time, OPN$^{-/-}$-hearts demonstrated with a significant up to 3.5-fold higher macrophage density in small, nontransmural infarctions when compared to their interstitial space (Figure 4(e)). The mRNA-expression of galectin-3, also known as MAC-2, was significantly induced after 3 and 5 days I/R in WT-hearts, as well as after 3 days in OPN$^{-/-}$-hearts, but not different between the two strains (Figure 4(f)). Interestingly, measurement of mRNA-expression of chemokines involved in this process revealed a significant 40% lower induction of macrophage-related CCL2 after 3 days I/R in OPN$^{-/-}$-hearts (Figure 4(g)). Another macrophage-related chemokine, CCL4, showed the same induction pattern in

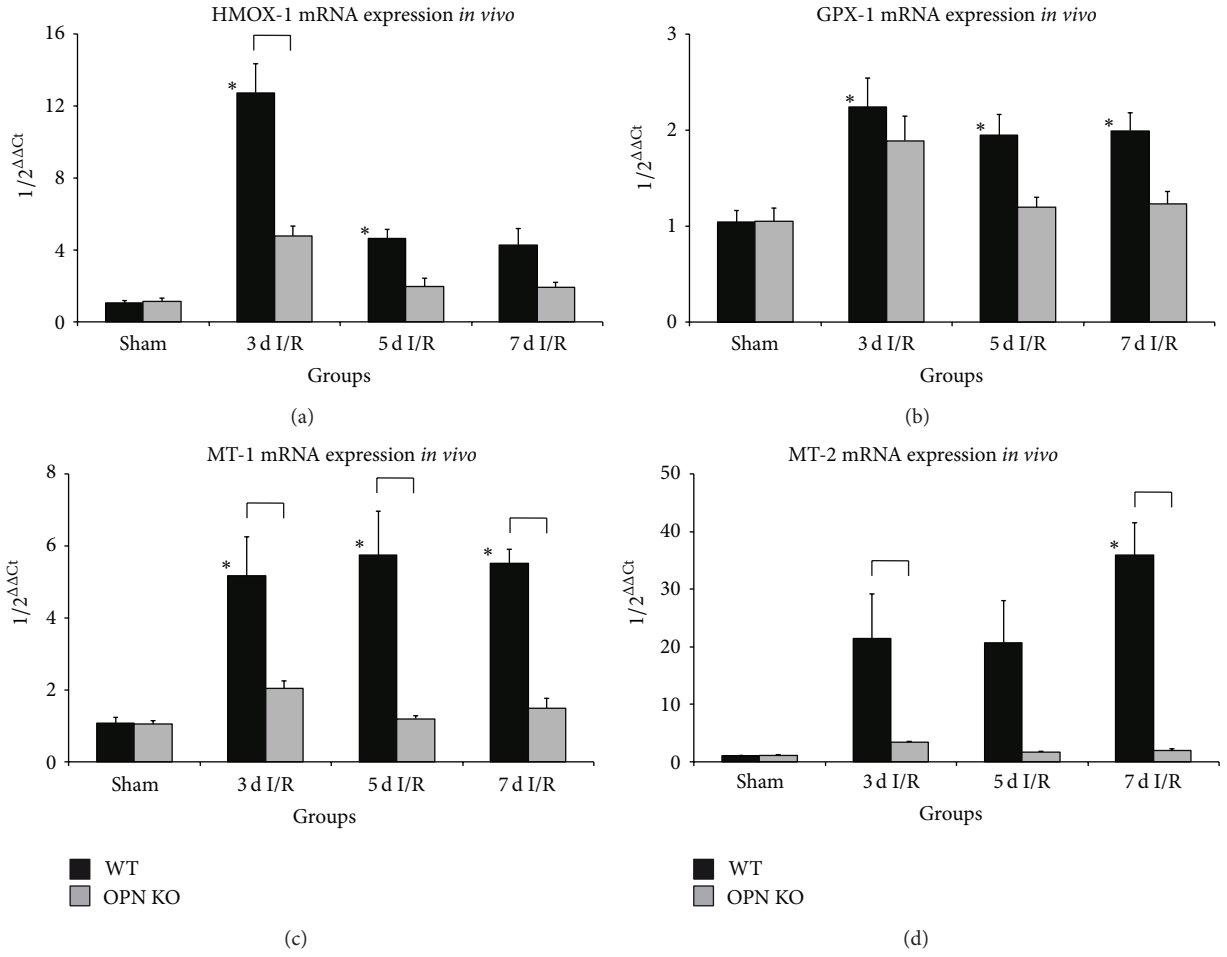


Figure 3: Ischemic OPN$^{-}/^{-}$-hearts show different expression of antioxidative mediators. mRNA-expression of antioxidative mediators (a) heme oxygenase- (HMOX-) 1 and (b) glutathione peroxidase- (GPX-) 1. mRNA-expression of zinc-storage proteins (c) metallothionein (MT)-1 and (d) MT-2. n = 8-9/group. RT-qPCR using Taqman and mRNA-expression is related to controls and GAPDH using comparative ΔΔCt-method. Bracket indicates $P \leq 0.05$ between genotypes; $*$ indicates $P \leq 0.05$ versus respective shams.

both genotypes as CCL2 (data not shown). Neutrophils-related chemokine CCL3 presented also with a significant 60% lower induction in OPN-hearts than in WT-hearts (Figure 4(h)), thus suggesting that chemokines are not the critical mediators driving the inflammatory response in osteopontin-deficient ischemic hearts. Indeed, the mRNA-expression of proinflammatory cytokine TNF-α showed a comparable induction between the genotypes (Figure 4(i)), thus suggesting that TNF-α acts as a main chemotactic agent in ischemic OPN$^{-}/^{-}$-hearts. The resolution of inflammatory response was evaluated using mRNA-expression of anti-inflammatory cytokine IL-10 (Figure 4(j)). IL-10 expression profile was comparable between both genotypes, and this provides suitable environment for the subsequent onset of myocardial remodeling.

3.4. Attenuated Remodeling in Ischemic OPN$^{-}/^{-}$-Hearts. We investigated several aspects of myocardial remodeling *in vivo* and analyzed the function of osteopontin in fibroblasts *in vitro*. Picrosirius red staining of collagen in WT-mice showed an evenly distributed interstitial fibrosis, with thin, dense collagen strains after 7 days I/R throughout the ischemic area of the left ventricle (Figure 5(a)). OPN$^{-}/^{-}$-hearts presented at the same time point with marked collagen deposition in small areas of nontransmural infarctions, which were partially confluent and where the collagen appeared not yet to be compacted (upper black arrow, Figure 5(b)). This morphology is indicative for prolonged remodeling process. In order to quantify the extent of these confluent, patchy, small areas of nontransmural infarctions, we performed planimetric analysis of picrosirius red area and found comparable total collagen stained area as a percentage of the total left ventricular area between both genotypes after 7 days I/R, which was significantly higher (up to 3-fold) than in the respective shams (Figure 5(c)). The differential analysis of collagen deposition in these small infarctions revealed a significantly larger area of them in OPN$^{-}/^{-}$-hearts covering almost 25% of the total left ventricular area, when compared to only 2% observed in WT-hearts (Figure 5(d)).

Further analysis of cells and remodeling markers included visualization of myofibroblasts using α-smac staining. In contrast to numerous myofibroblasts found in ischemic area

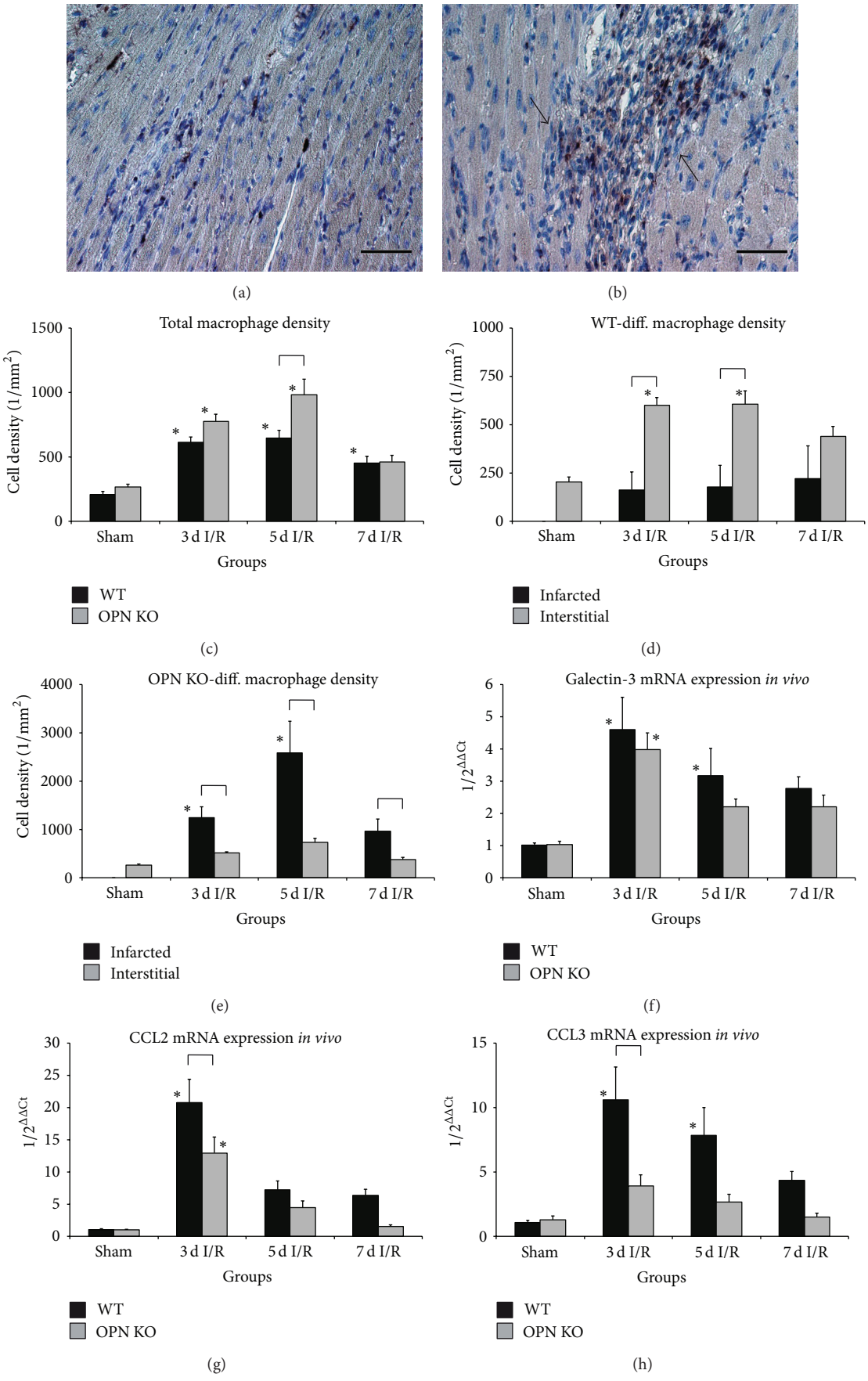


Figure 4: Continued.

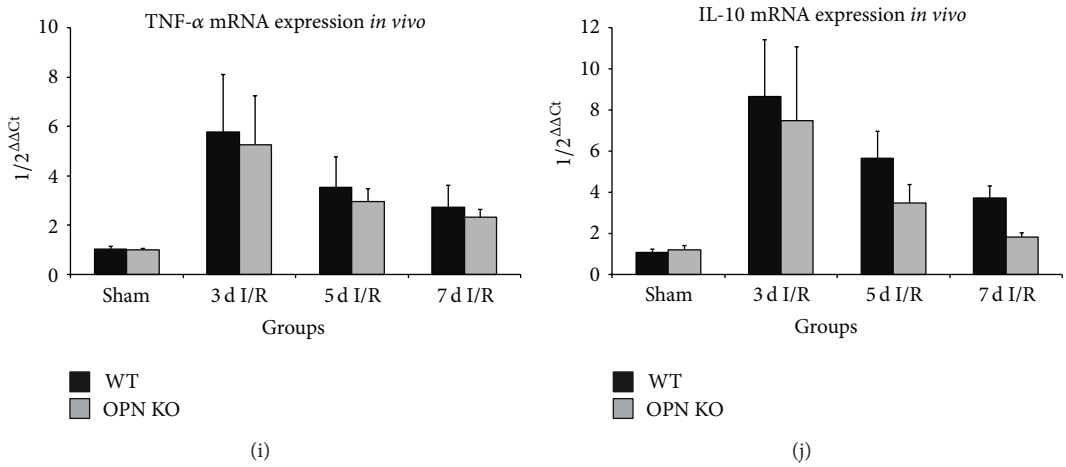


FIGURE 4: Cellular and molecular changes in response to I/R. (a) Representative section of left ventricle after 7 days I/R in a WT-heart shows low interstitial macrophage accumulation (F4/80). (b) High accumulation of macrophages in areas of small nontransmural infarctions in OPN^{-}/$^{-}$-hearts (arrows). (c) Cell density of F4/80 positive macrophages in WT- compared to OPN^{-}/$^{-}$-hearts during I/R. (d) Differential macrophage cell count in WT-hearts revealed only few cells within areas of small infarctions (infarcted) in comparison to interstitial space. (e) In contrast, significantly more cells invaded areas of small infarctions in OPN^{-}/$^{-}$-mice than interstitial space. mRNA-induction of the galectin-3 (f), chemokines (g) CCL2 and (h) CCL3, and cytokines (i) TNF-α and (j) IL-10 in OPN^{-}/$^{-}$-hearts compared to WT-hearts during repetitive I/R. n = 8–11/group. Scale bars represent 50 μm. RT-qPCR using Taqman and mRNA-expression is related to controls and GAPDH using comparative ΔΔCt-method. Bracket indicates $P \leq 0.05$ between genotypes; $*$ indicates $P \leq 0.05$ versus respective shams.

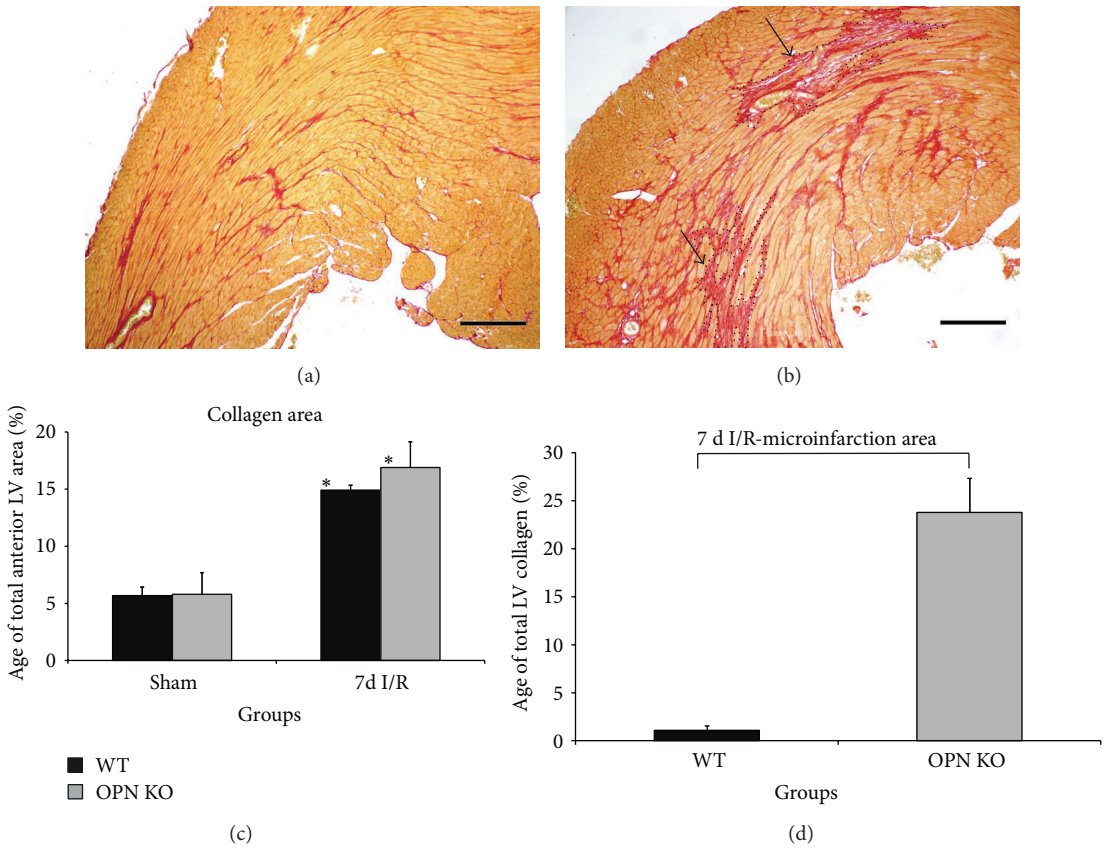


FIGURE 5: Collagen deposition in areas of small, nontransmural infarctions in OPN^{-}/$^{-}$-hearts during repetitive I/R. (a) Representative section of left ventricle in a WT-heart after 7 days I/R shows interstitial fibrosis in picrosirius red staining. (b) In contrast, OPN^{-}/$^{-}$-mice reveal relatively loose collagen deposition in areas of cardiomyocyte loss-small, nontransmural infarctions (arrows and dotted lines show confluent areas of patchy replacement fibrosis), but less interstitial collagen in the ischemic area. (c) Planimetrical analysis of collagen stained area between the genotypes after 7 days I/R. (d) Differential analysis of collagen deposition in small, nontransmural infarctions. n = 8–11/group. Scale bars represent 200 μm. Bracket indicates $P \leq 0.05$ between genotypes; $*$ indicates $P \leq 0.05$ versus respective shams.

of the WT-myocardium (Figure 6(a)), we observed only very few myofibroblasts in ischemic OPN^{-}/$^{-}$-hearts (Figure 6(b)). The mRNA-expression of early remodeling marker tenascin-C was strongly induced in WT-hearts being ~18-fold higher than in OPN^{-}/$^{-}$-hearts (Figure 6(c)). Still, the OPN^{-}/$^{-}$-hearts presented with ~4-fold induction above the sham-level. The mRNA-expression of growth and myofibroblast differentiation factor TGF-β1 was comparable between the genotypes and slightly induced in both strains after 3 days I/R (data not shown). Another prominent and significant difference was found in the absence of induction of MMP-2 and MMP-9 in OPN^{-}/$^{-}$-hearts when compared to their induction in WT-samples (Figures 6(d) and 6(e)). Interestingly, MMP-12 expression was also significantly ~4-fold higher in WT-hearts after 3 days I/R when compared to the OPN^{-}/$^{-}$-hearts, but the OPN^{-}/$^{-}$-hearts revealed a nonsignificant ~2-fold induction when compared to their shams (Figure 6(f)). Apparently, the only enzyme involved in collagen deposition was MMP-13, which was significantly up to 3-fold higher induced in OPN^{-}/$^{-}$-hearts after 3 days I/R and followed by a tendency to a higher expression thereafter, when compared to the WT-hearts (Figure 6(g)). Interestingly, the expression of tissue-inhibitor of MMP (TIMP-1) (Figure 6(h)) and TIMP-2 (Figure 6(i)) was also significantly lower in OPN^{-}/$^{-}$-hearts during repetitive I/R than in WT-hearts. TIMP-4 presented a similar induction pattern as TIMP-2 for both genotypes (data not shown).

In the last set of experiments we analyzed fibroblasts *in vitro*, which were derived from naïve hearts, cultured under normoxic or hypoxic conditions, and additionally stimulated with human TGF-β1 to differentiate into myofibroblasts. As a proof of principle, we measured the OPN-1 in WT-cells and found a significantly lower expression of it under hypoxia with or without TGF-β1 stimulation (Figure 7(a)). The 24 h stimulation led further to significantly less induction of OPN-1 under normoxic conditions. Therefore, fibroblasts seem to downregulate OPN-1 *in vitro* and this may represent a negative feedback for the expression of OPN-1 during the later phases of myocardial remodeling, when extracellular matrix production is slowing down. The mRNA-expression of galectin-3 showed no differences between the strains or conditions, thus indicating no role for it in fibroblasts (Figure 7(b)). WT-myofibroblasts presented dynamic regulation of CCL2 with a significant ~4-fold higher mRNA-induction after 6 h of TGF-β1 stimulation irrespectively of hypoxic condition (Figure 7(c)). This induction was not observed after 24 h, while OPN^{-}/$^{-}$-hearts presented no induction of CCL2 under any of conditions. The mRNA-expression of tenascin-C was significantly induced after 6 and 24 h TGF-β1 stimulation and normoxia in WT-fibroblasts (Figure 7(d)). In addition, hypoxia led to a significant induction of tenascin-C mRNA after 24 h stimulation in the WT-cells. OPN^{-}/$^{-}$-hearts presented again with an absence of significant mRNA-induction of tenascin-C under any of the investigated conditions. Similar to the *in vivo* findings we observed a tendency to a higher expression of MMP-9 after 24 h TGF-β1 stimulation in WT-mice under hypoxia when compared to OPN^{-}/$^{-}$-cells, while this difference reached a significant level in stimulated group under normoxic conditions (Figure 7(e)). In contrast to the *in vivo* data, we found no significant induction of MMP-12 mRNA *in vitro* (Figure 7(f)). Still, another parallel to the *in vivo* data was observed in mRNA-expression of TIMP-1. TIMP-1 expression was significantly higher after 24 h TGF-β1 stimulation in myofibroblasts from WT-hearts under normoxic as well as hypoxic atmosphere when compared to no induction in OPN^{-}/$^{-}$-cells (Figure 7(g)).

Taken together, these data provide novel evidence for an osteopontin-mediated, differential regulation of mediators in myocardial remodeling in a model of murine ischemic cardiomyopathy.

4. Discussion

This study provides novel insights into cardioprotective mechanisms of osteopontin in our murine model of brief, repetitive I/R in absence of myocardial infarction. It contributes to a better understanding of the previously described role for osteopontin in regulation of macrophage function and myocardial fibrosis after infarction [6]. Osteopontin has also been associated with development of fibrosis and myocardial adaptation in an angiotensin II-induced murine hypertrophy model [9]. Mice with a cardiomyocyte specific overexpression of osteopontin died prematurely after mean of 12 weeks of age. In these mice, loss of cardiomyocytes was associated with increased inflammatory cell infiltration and their cytotoxic activity [11]. On the other hand, lack of osteopontin caused increased apoptosis of cardiomyocytes in *streptozotocin*-induced diabetic mice [16]. Further investigations showed expression of osteopontin on noncardiomyocytes in a desmin-deficient mouse model suffering from myocardial degeneration in absence of any kind of injury [12]. Expression of osteopontin has been shown on neonatal rat cardiomyocytes [17]. In the present study, we found that osteopontin is specifically induced in murine cardiomyocytes of WT-mice after repetitive ischemic insults. Our OPN^{-}/$^{-}$-mice presented with loss of cardiomyocytes and deteriorated left ventricular function after 7 days repetitive I/R. This was associated with changes in mRNA-expression of contractile elements. OPN^{-}/$^{-}$-mice did not change their utilization of the less energy efficient α-MHC isoform despite repetitive ischemic injury, but were still able to adapt by induction of the less ATP consuming β-MHC isoform. Even though this resulted in a comparable ratio of β/α-MHC between the WT and OPN^{-}/$^{-}$-mice, the increase in metabolic turnover in OPN^{-}/$^{-}$-mice may represent an additional burden under ischemic stress. The reexpression of embryonic contractile elements has been associated with different myocardial pathologies [18, 19]. Our findings on expression of skeletal and cardiac actin, as well as desmin in OPN^{-}/$^{-}$-mice, give further support to an osteopontin-mediated adaptation of cardiomyocytes to ischemic injury.

Osteopontin has been described to regulate expression of heme oxygenase-1 in glioma cells [20]. We found lower induction of heme oxygenase-1 and absent induction of glutathione peroxidase-1, as well as only minimal induction of metallothionein-1 and -2, in OPN^{-}/$^{-}$-mice, which all reflect functional impairment of antioxidative mediators as very

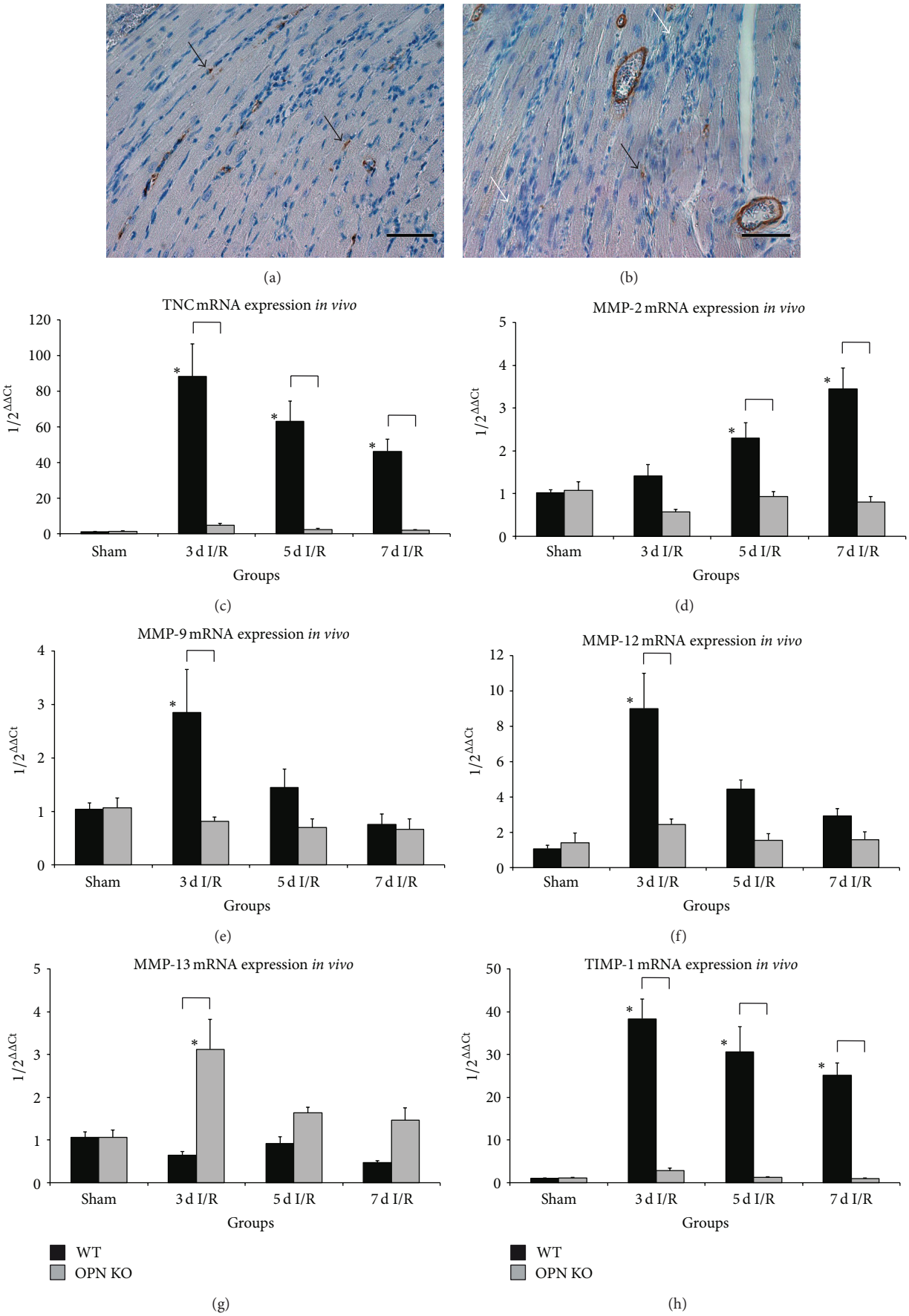
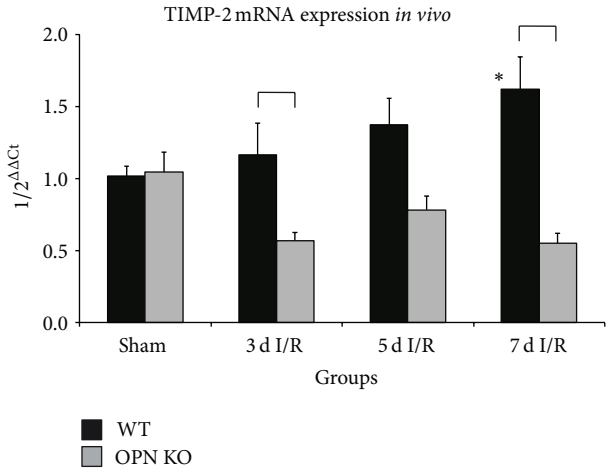


Figure 6: Continued.



(i)

Figure 6: Attenuated remodeling in ischemic OPN$^{-}/^{-}$-hearts. (a) Representative section of left ventricle after 7 days I/R in a WT-heart shows numerous α-smac positive myofibroblasts (black arrows) in the ischemic myocardium. (b) At the same time point, α-smac positive signals (black arrow) were almost absent in small, nontransmural infarctions (white arrows) of OPN$^{-}/^{-}$-hearts. mRNA-induction of (c) tenascin-C (TNC), (d) MMP-2, (e) MMP-9, (f) MMP-12, (g) MMP-13, (h) TIMP-1, and (i) TIMP-2 in WT- and OPN$^{-}/^{-}$-hearts during repetitive I/R. $n = 8$–11/group. Scale bars represent 50 μm. RT-qPCR using Taqman and mRNA-expression is related to controls and GAPDH using comparative $\Delta\Delta$Ct-method. Bracket indicates $P \leq 0.05$ between genotypes; $*$ indicates $P \leq 0.05$ versus respective shams.

important cardioprotective mechanism [4]. The lack of metallothionein induction in OPN$^{-}/^{-}$-mice is very interesting, since these zinc-storage proteins are important for normal function of several enzymes, antioxidative mediators, and actors in inflammatory reaction [21]. Taken together, our findings on contractile elements expression and antioxidative mediators support the specific role of osteopontin on cardiomyocytes.

According to previously published data on the role of osteopontin in macrophage function [6, 22], we found significantly stronger macrophage infiltration of the ischemic myocardium in OPN$^{-}/^{-}$-mice. In contrast to previous studies, we demonstrated predominant attraction of invading macrophages to the small, nontransmural infarctions thereby contributing to the phagocytosis of dead cardiomyocytes and subsequent scar formation. Another interesting finding of our study is the lower mRNA-induction of chemokines CCL2 and CCL3 in OPN$^{-}/^{-}$-mice during repetitive I/R. Our *in vivo* data is also supported by the lack of CCL2-induction found in our myofibroblast cell culture. These findings are novel and additive in respect to a study, where osteopontin has been suggested to specifically modulate expression of CCL2 chemokine and thus regulating activity of monocytes *in vitro* [23]. Therefore, our findings may be also interpreted as a result of osteopontin-deficiency on noncardiomyocytes, that is, macrophages and fibroblasts in the heart. But one may still argue that the lack of antioxidative mediators in cardiomyocytes could be responsible for the observed lower induction of chemokines [24]. Therefore, we can only remain speculative in this matter, and this question could be addressed using cell-specific knockouts in future studies. Despite the differences in chemokine expression, OPN$^{-}/^{-}$-mice showed normal cytokine expression during repetitive I/R, which was sufficient to mediate the observed macrophage infiltration. The resolution of inflammatory response, demonstrated by normal IL-10 mRNA-induction, also seems to be unaffected by osteopontin-deficiency, thus allowing the timely onset of myocardial remodeling. A recent study provided evidence for a substantial role of osteopontin on noncardiomyocytes, that is, macrophages via upregulation of galectin-3 and MMP-12 during myocardial degeneration of desmin-deficient mice without injury [12]. We did not find a difference in galectin-3 expression in OPN$^{-}/^{-}$-hearts, but this is probably attributable to the fact that our model of repetitive ischemic injury does not induce a substantial damage to the myocardium.

Numerous studies reported involvement of osteopontin in mechanisms of tissue remodeling. Osteopontin-expression was necessary to induce mediators of fibrosis in development of *bleomycin*-induced lung fibrosis [7]. Mice lacking osteopontin showed exaggerated left ventricular dilation and presented with reduced collagen deposition after myocardial infarction without reperfusion [10]. In contrast, we observed somewhat opposite findings with comparable total collagen area between WT- and OPN$^{-}/^{-}$-mice after 7 days I/R, but high collagen deposition in small infarctions associated with less interstitial fibrosis in the OPN$^{-}/^{-}$-mice. The collagen fibers seem not to be compacted after 7 days I/R in OPN$^{-}/^{-}$-mice, which can indicate an impaired myocardial remodeling during formation of a stable scar with compacted collagen fibers. Since osteopontin was described to be necessary for differentiation of myofibroblasts *in vitro* [8], we examined the differentiation of myofibroblasts *in vivo* and found less of these collagen-producing cells in OPN$^{-}/^{-}$-mice. Tenascin-C is an established early marker of remodeling and is also strongly involved during embryonic development [25] or scar formation after myocardial infarction [26]. In the present study, we describe for the first time that tenascin-C expression is regulated by osteopontin in myocardial

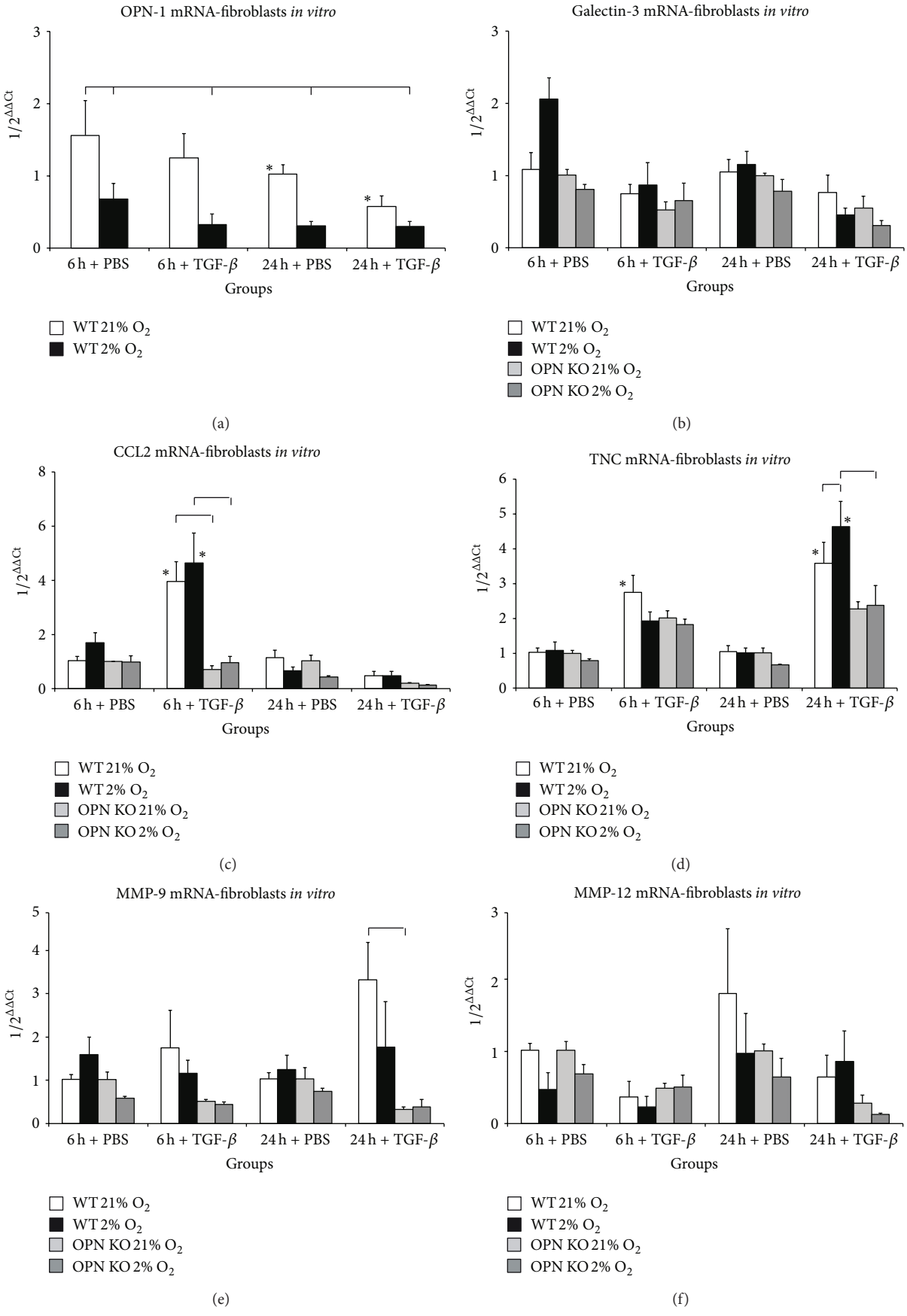
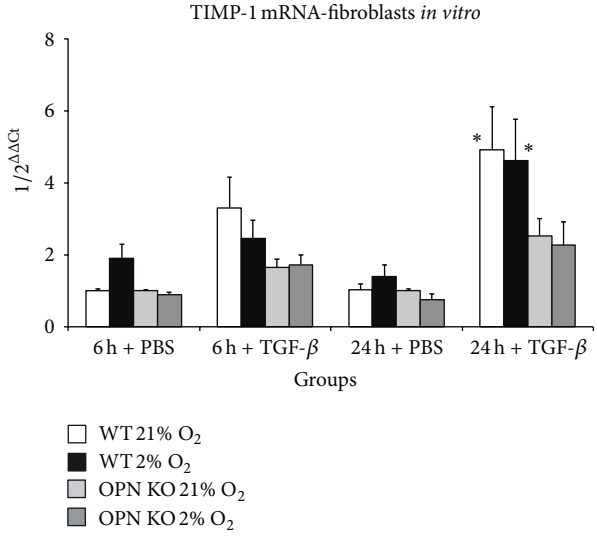


Figure 7: Continued.



(g)

Figure 7: Expression of remodeling markers in myofibroblasts *in vitro*. Cultured myofibroblasts from WT- and OPN$^{-}/^{-}$-hearts were stimulated with human TGF-β1 or PBS as control under normoxia (21% O_2) or hypoxia (2% O_2). mRNA-expression of (a) OPN-1 in WT-cells only. mRNA-expression of (b) galectin-3, (c) CCL2, (d) tenascin-C (TNC), (e) MMP-9, (f) MMP-12, and (g) TIMP-1. n = 5–7/group. RT-qPCR using Taqman and mRNA-expression is related to controls and 18 S (*in vitro*) using comparative ΔΔCt-method. Bracket indicates $P \leq 0.05$ between genotypes; $*$ indicates $P \leq 0.05$ versus 6 h + PBS normoxia group (control).

remodeling *in vivo* and *in vitro*, thereby contributing to timely onset of the remodeling process. In addition to the morphological findings on less compacted collagen in small infarctions, these data clearly indicate impaired remodeling in OPN$^{-}/^{-}$-hearts. Osteopontin has also been described as an important regulator of MMPs and their tissue inhibitors during remodeling resulting in significantly decreased left ventricular dilation after myocardial infarction [27]. We could confirm these data showing a decrease in mRNA-expression of MMP-2, MMP-9, MMP-12, TIMP-1, TIMP-2, and TIMP-4 *in vivo*. In addition, we found an increase in MMP-13 expression in OPN$^{-}/^{-}$-mice, being probably one of a few factors mediating the collagen deposition and scar formation during repetitive I/R in these mice. Our data from myofibroblasts *in vitro* showed a decrease in osteopontin expression under hypoxia and TGF-β stimulation. Therefore, osteopontin seems to be rather involved during transition from macrophage-driven inflammation to early remodeling phase, since it has also been suggested as a marker for mature macrophages during late stages of granulation tissue formation [28]. The missing upregulation of galectin-3 and MMP-12 *in vitro* also points to their predominant role on macrophages, as previously suggested [12]. Still, the *in vitro* MMP-9 and TIMP-1 data are supportive for our *in vivo* findings. Therefore, our data provide also evidence for the role of osteopontin in regulation of chemokines and macrophage function and subsequent effects in remodeling during development of murine ischemic cardiomyopathy.

Our experimental study may also have a clinical relevance based on recently published human data. Osteopontin-expression was increased on cardiomyocytes in patients with terminal heart failure [29], and its level has been suggested as a predictive marker of development of right ventricular failure [30]. Also, osteopontin has been suggested as a marker of mortality and readmission risk in patients with acute congestive heart failure [31]. In this respect, we emphasize that the interpretation of our data may have a weakness being solely based on mRNA-expression without protein data. Still, this is somewhat relative due to the fact that many of our reported factors are transcriptionally well regulated.

5. Conclusion

In conclusion, osteopontin seems to modulate expression of contractile elements, antioxidative enzymes, inflammatory response, and early development of interstitial fibrosis in order to prevent irreversible cardiomyocyte loss in murine ischemic cardiomyopathy. These findings may further support the therapeutical perspective for osteopontin as a possible target in protection of the ischemic heart.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors thank Susan Rittling, Rutgers University, Piscataway, NJ, USA, for providing them with a breeding pair of OPN$^{-}/^{-}$-mice. The authors also thank Christine Peigney for expert assistance in immunohistochemistry, Helge Dörr for expert assistance in molecular biology and animal surgery, Frank Holst for performing the Langendorff-perfusion, Sven

Wehner for technical assistance in cell culture, and Alexander von Ruecker for providing RT-qPCR equipment. This work was supported by BONFOR grants from the Medical School, University of Bonn (to G.D.D. and O.D.).

References

[1] R. A. Kloner, R. Bolli, E. Marban, L. Reinlib, and E. Braunwald, "Medical and cellular implications of stunning, hibernation, and preconditioning: an NHLBI workshop," *Circulation*, vol. 97, no. 18, pp. 1848–1867, 1998.

[2] N. G. Frangogiannis, S. Shimoni, S. Chang et al., "Active interstitial remodeling: an important process in the hibernating human myocardium," *Journal of the American College of Cardiology*, vol. 39, no. 9, pp. 1468–1474, 2002.

[3] N. G. Frangogiannis, S. Shimoni, S. M. Chang et al., "Evidence for an active inflammatory process in the hibernating human myocardium," *American Journal of Pathology*, vol. 160, no. 4, pp. 1425–1433, 2002.

[4] O. Dewald, N. G. Frangogiannis, M. Zoerlein et al., "Development of murine ischemic cardiomyopathy is associated with a transient inflammatory reaction and depends on reactive oxygen species," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 5, pp. 2700–2705, 2003.

[5] N. G. Frangogiannis, O. Dewald, Y. Xia et al., "Critical role of monocyte chemoattractant protein-1/CC chemokine ligand 2 in the pathogenesis of ischemic cardiomyopathy," *Circulation*, vol. 115, no. 5, pp. 584–592, 2007.

[6] D. T. Denhardt, M. Noda, A. W. O'Regan, D. Pavlin, and J. S. Berman, "Osteopontin as a means to cope with environmental insults: regulation of inflammation, tissue remodeling, and cell survival," *Journal of Clinical Investigation*, vol. 107, no. 9, pp. 1055–1061, 2001.

[7] J. S. Berman, D. Serlin, X. Li et al., "Altered bleomycin-induced lung fibrosis in osteopontin-deficient mice," *American Journal of Physiology: Lung Cellular and Molecular Physiology*, vol. 286, no. 6, pp. L1311–L1318, 2004.

[8] Y. Lenga, A. Koh, A. S. Perera, C. A. McCulloch, J. Sodek, and R. Zohar, "Osteopontin expression is required for myofibroblast differentiation," *Circulation Research*, vol. 102, no. 3, pp. 319–327, 2008.

[9] Y. Matsui, N. Jia, H. Okamoto et al., "Role of osteopontin in cardiac fibrosis and remodeling in angiotensin II-induced cardiac hypertrophy," *Hypertension*, vol. 43, no. 6, pp. 1195–1201, 2004.

[10] N. A. Trueblood, Z. Xie, C. Communal et al., "Exaggerated left ventricular dilation and reduced collagen deposition after myocardial infarction in mice lacking osteopontin," *Circulation Research*, vol. 88, no. 10, pp. 1080–1087, 2001.

[11] M.-A. Renault, F. Robbesyn, P. Réant et al., "Osteopontin expression in cardiomyocytes induces dilated cardiomyopathy," *Circulation: Heart Failure*, vol. 3, no. 3, pp. 431–439, 2010.

[12] S. Psarras, M. Mavroidis, D. Sanoudou et al., "Regulation of adverse remodelling by osteopontin in a genetic heart failure model," *European Heart Journal*, vol. 33, no. 15, pp. 1954–1963, 2012.

[13] S. R. Rittling, H. N. Matsumoto, M. D. Mckee et al., "Mice lacking osteopontin show normal development and bone structure but display altered osteoclast formation in vitro," *Journal of Bone and Mineral Research*, vol. 13, no. 7, pp. 1101–1111, 1998.

[14] J. W. Schrickel, K. Brixius, C. Herr et al., "Enhanced heterogeneity of myocardial conduction and severe cardiac electrical instability in annexin A7-deficient mice," *Cardiovascular Research*, vol. 76, no. 2, pp. 257–268, 2007.

[15] P. Huebener, T. Abou-Khamis, P. Zymek et al., "CD44 is critically involved in infarct healing by regulating the inflammatory and fibrotic response," *Journal of Immunology*, vol. 180, no. 4, pp. 2625–2633, 2008.

[16] V. Subramanian, P. Krishnamurthy, K. Singh, and M. Singh, "Lack of osteopontin improves cardiac function in streptozotocin-induced diabetic mice," *American Journal of Physiology: Heart and Circulatory Physiology*, vol. 292, no. 1, pp. H673–H683, 2007.

[17] K. Graf, Y. S. Do, N. Ashizawa et al., "Myocardial osteopontin expression is associated with left ventricular hypertrophy," *Circulation*, vol. 96, no. 9, pp. 3063–3071, 1997.

[18] R. B. Driesen, F. K. Verheyen, W. Debie et al., "Re-expression of alpha skeletal actin as a marker for dedifferentiation in cardiac pathologies," *Journal of Cellular and Molecular Medicine*, vol. 13, no. 5, pp. 896–908, 2009.

[19] T. Omura, M. Yoshiyama, K. Takeuchi et al., "Differences in time course of myocardial mRNA expression in non-infarcted myocardium after myocardial infarction," *Basic Research in Cardiology*, vol. 95, no. 4, pp. 316–323, 2000.

[20] D.-Y. Lu, W.-L. Yeh, S.-M. Huang, C.-H. Tang, H.-Y. Lin, and S.-J. Chou, "Osteopontin increases heme oxygenase-1 expression and subsequently induces cell migration and invasion in glioma cells," *Neuro-Oncology*, vol. 14, no. 11, pp. 1367–1378, 2012.

[21] D. U. Spahl, D. Berendji-Grün, C. V. Suschek, V. Kolb-Bachofen, and K.-D. Kröncke, "Regulation of zinc homeostasis by inducible NO synthase-derived NO: nuclear metallothionein translocation and intranuclear Zn^{2+} release," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 2, pp. 13952–13957, 2003.

[22] V. Ophascharoensuk, C. M. Giachelli, K. Gordon et al., "Obstructive uropathy in the mouse: role of osteopontin in interstitial fibrosis and apoptosis," *Kidney International*, vol. 56, no. 2, pp. 571–580, 1999.

[23] J. Sun, A. Feng, S. Chen et al., "Osteopontin splice variants expressed by breast tumors regulate monocyte activation via MCP-1 and TGF-β1," *Cellular and Molecular Immunology*, vol. 10, no. 2, pp. 176–182, 2013.

[24] Y. Zheng, M. Toborek, and B. Hennig, "Epigallocatechin gallate-mediated protection against tumor necrosis factor-α-induced monocyte chemoattractant protein-1 expression is heme oxygenase-1 dependent," *Metabolism: Clinical and Experimental*, vol. 59, no. 10, pp. 1528–1535, 2010.

[25] M. Flück, S. I. Mund, J. C. Schittny, S. Klossner, A.-C. Durieux, and M.-N. Giraud, "Mechano-regulated tenascin-C orchestrates muscle repair," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 36, pp. 13662–13667, 2008.

[26] O. Dewald, G. D. Duerr, N. Elhafi et al., "Comparison of myocardial remodeling between cryoinfarction and reperfused infarction in mice," *Journal of Biomedicine and Biotechnology*, vol. 2011, Article ID 961298, 10 pages, 2011.

[27] P. Krishnamurthy, J. T. Peterson, V. Subramanian, M. Singh, and K. Singh, "Inhibition of matrix metalloproteinases improves left ventricular function in mice lacking osteopontin after myocardial infarction," *Molecular and Cellular Biochemistry*, vol. 322, no. 1-2, pp. 53–62, 2009.

[28] C. E. Murry, C. M. Giachelli, S. M. Schwartz, and R. Vracko, "Macrophages express osteopontin during repair of myocardial necrosis," *American Journal of Pathology*, vol. 145, no. 6, pp. 1450–1462, 1994.

[29] P. Stawowy, F. Blaschke, P. Pfautsch et al., "Increased myocardial expression of osteopontin in patients with advanced heart failure," *European Journal of Heart Failure*, vol. 4, no. 2, pp. 139–146, 2002.

[30] T. S. Kato, A. Chokshi, P. Singh et al., "Markers of extracellular matrix turnover and the development of right ventricular failure after ventricular assist device implantation in patients with advanced heart failure," *Journal of Heart and Lung Transplantation*, vol. 31, no. 1, pp. 37–45, 2012.

[31] M. Behnes, M. Brueckmann, S. Lang et al., "Diagnostic and prognostic value of osteopontin in patients with acute congestive heart failure," *European Journal of Heart Failure*, vol. 15, no. 12, pp. 1390–1400, 2013.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 206026, 12 pages
http://dx.doi.org/10.1155/2014/206026

Research Article

IL-6 Impairs Myogenic Differentiation by Downmodulation of p90RSK/eEF2 and mTOR/p70S6K Axes, without Affecting AKT Activity

Michele Pelosi,[1] Manuela De Rossi,[1] Laura Barberi,[1] and Antonio Musarò[1,2]

[1] *Institute Pasteur Cenci-Bolognetti, DAHFMO-Unit of Histology and Medical Embryology, IIM, Sapienza University of Rome, Via A. Scarpa 16, 00161 Rome, Italy*
[2] *Center for Life Nano Science@Sapienza, Istituto Italiano di Tecnologia, Viale Regina Elena 291, 00161 Rome, Italy*

Correspondence should be addressed to Antonio Musarò; antonio.musaro@uniroma1.it

Received 7 February 2014; Accepted 2 May 2014; Published 21 May 2014

Academic Editor: Pura Muñoz-Cánoves

IL-6 is a multifaceted pleiotropic cytokine, which is produced by a variety of cell types and targets different cells and tissues. In physiological conditions, IL-6 can be locally and transiently produced by skeletal muscle and plays an important role in muscle homeostasis. Circulating IL-6 levels are normally very low or undetectable but are dramatically increased in several pathologic conditions. In this study, we aimed to define the potential molecular mechanisms underlying the effects of IL-6 on myogenic program. We explored the molecular mechanisms through which exogenous IL-6, or the conditioned medium from the murine C-26 adenocarcinoma cells (a cellular model that secretes high levels of IL-6 and induces cancer cachexia in mice), interferes with the myogenic program. Our study revealed that IL-6 induces the activation of the Stat3 signaling and promotes the downmodulation of the p90RSK/eEF2 and mTOR/p70S6K axes, while it does not affect the activation of AKT. We thus identified potential molecular mediators of the inhibitory effects of IL-6 on myogenic program.

1. Introduction

Muscle differentiation is a well-coordinated and regulated process which is influenced by either positive or negative external signals. Fibroblast growth factor (FGF) and TGF-β inhibit differentiation [1, 2], whereas IGFs are potent inducers of myogenic proliferation, differentiation, and hypertrophy [3, 4].

Moreover, several cytokines and other factors, such as IL-1, IL-6, TNF-α, and IFN-γ, have been proven to negatively affect muscle differentiation both in vitro and in vivo, causing also muscle wasting [5–8]. Elevated levels of cytokines, associated with chronic inflammation, are observed in several chronic diseases, ranging from cancer to AIDS and from muscular dystrophy to chronic heart failure and kidney disease.

Among these factors, IL-6 potentially plays a prominent role in muscle differentiation, homeostasis, and wasting.

IL-6 can be produced locally, by skeletal muscle, or released systemically in response to different physiopathologic stimuli [9]. In skeletal muscle IL-6 acts as a pleiotropic factor and appears to have at least two different conflicting functions. In nonpathological conditions, IL-6 has been involved in the physiological metabolic response of skeletal muscle to exercise [9, 10]; it is synthesized by skeletal muscle, in response to increased workload, and secreted locally or into the blood stream, where it might act in a hormone-like manner, stimulating the hepatic gluconeogenesis and increasing adipose tissue lipolysis [10]. It has been also shown that the autocrine production of IL-6 can promote myogenic differentiation [11, 12] and acts as a positive regulator of satellite cell-mediated hypertrophic muscle growth [13], suggesting that IL-6 plays an important role in myogenesis.

By contrast, different pathologic conditions promoting muscle wasting, including cancer, are associated with elevated systemic levels of IL-6 [4]. Moreover, severe muscle atrophy

is seen in different mouse models in which IL-6 has been chronically elevated in skeletal muscle, through transgenic overexpression [14], in vivo electroporation [15], or direct infusion into muscle [16]. The different and opposite effects of a single molecule, like IL-6, can be explained taking into consideration the levels of expression and its spatial and temporal interaction with the target tissue. The regulation of muscle differentiation is dependent on the activation of signal transduction cascades with the complex involvement of several kinases, including the mitogen-activated protein kinases (MAPK), the serine/threonine kinase AKT, a downstream effector of PI3-kinase, the p90 ribosomal S6 kinase (p90RSK), and the calcineurin/NFAT pathway [17].

In this study, we explored some molecular mechanisms through which exogenous IL-6 can perturb the skeletal muscle homeostasis and impinge muscle differentiation.

We treated C2C12 myogenic cells, an in vitro model able to recapitulate the program of myogenic differentiation with recombinant IL-6 or with the conditioned medium from the C-26 mouse adenocarcinoma cell line (C-26 CM), which secrete IL-6 in the extracellular medium (as it is shown in this paper and in [5, 18]). We show that IL-6 induced a dramatic inhibition in the expression of late myogenic differentiation markers, such as myogenin and sarcomeric muscle myosin, whereas it did not significantly affect the expression of Pax7 and MyoD. IL-6 also did not affect the activation of AKT, a kinase essential to promote protein synthesis and cell survival and to block protein degradation [19, 20]. However, we show that exogenous IL-6 induced a selective downmodulation of the p90RSK/eEF2 and mTOR/p70S6K axes, which are important mediators for the positive control of protein synthesis and the maintenance of the differentiated myogenic phenotype [19, 21–23].

2. Material and Methods

2.1. Cell Culture and Media Preparation. Murine C2C12 myoblasts cells and C-26 colon adenocarcinoma (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum plus standard complements (growth medium, GM). To induce myogenic differentiation of C2C12 myoblasts, cells were cultured in GM until subconfluence (i.e., about 80–90% of cell confluence) and then shifted to DMEM with 2% horse serum (differentiation medium, DM). All media, sera, and complements for cell cultures were from Gibco-Invitrogen. For C-26 conditioned medium (CM) production, about 8×10^5 C-26 cells were plated in GM in a 60×15 mm cell culture dish (Falcon). After the overnight attachment, the medium was totally replaced with 6 mL of fresh GM. When the C-26 cells reached confluence, the resulting medium (C-26 CM) was collected, centrifuged (1000 g for 10 min), and filtered with a 0.22 μm polyethersulfone hydrophilic filter (Millipore). 2% horse serum (final concentration) was added to C-26 CM, which was finally diluted in a ratio 1 : 10 in the DM, to obtain the DM + 10% C-26 CM medium.

Recombinant mouse IL-6 (rIL-6) was from R&D Systems. Lyophilized rIL-6 was dissolved at 100 μg/mL in sterile PBS containing 0.1% bovine serum albumin (BSA, globulin-free and low endotoxin, purity ≥98%, from Sigma). rIL-6 was diluted into DM to obtain the (DM + 25 ng/mL of rIL-6) medium. The same amount of carrier BSA was added in the control DM. However, previous analysis showed that the carrier BSA did not interfere with myogenic differentiation (data not shown).

2.2. Cytokine Assay. The BD Biosciences CBA Mouse Inflammation Kit was used to quantitatively measure IL-6, IL-10, MCP-1, IFN-γ, TNF-α, and IL-12p70 protein levels, according to the manufacturer instructions. In brief, 50 μL of supernatants (C-26 CM or control samples) were incubated with a mixture of beads coated with the capture antibodies for the different cytokines. Addition of the PE-conjugated detection antibodies formed a sandwich complex. After 2 h of incubation and one wash, samples were analyzed by flow cytometry, using an Epics XL Cytometer (Beckman Coulter). Standard curves were generated from analysis of titrated cytokine standards (at least 10 serial dilutions; standards provided by the manufacturer) and analysis was performed using the BD CBA analysis software.

2.3. Immunohistochemical Analysis. C2C12 myotubes were fixed in 4% paraformaldehyde and then preincubated in phosphate-buffered saline (PBS) containing 1% BSA and a 1 : 30 dilution of goat serum for 30 min. Myotubes were treated with a monoclonal antibody against the anti-myosin type II (MF-20, Developmental Studies Hybridoma Bank) (1 : 100, overnight at 4°C). After incubation with the primary antibody, the myotubes were washed twice with PBS and once with PBS containing 1% BSA for 15 min and incubated with goat anti-mouse rhodamine-conjugated secondary antibody. The cells were washed again, as described above, and the nuclei of myogenic cells were visualized by Hoechst staining. Finally the slides were mounted in 90% glycerol in PBS (pH 8) and visualized under inverted microscope (Axioskop 2 plus; Carl Zeiss MicroImaging, Inc.) and images were processed using Axiovision 3.1.

2.4. Protein Extraction and Immunoblotting. C2C12 cells were washed with PBS and lysed in RIPA buffer (radio immunoprecipitation assay buffer) (100 mM Tris/HCl pH 7.4, 150 mM NaCl, 20 mM sodium fluoride, 2 mM EDTA, 0.2 mM 2-glycerophosphate, 1 mM sodium vanadate, 3% (w/v) deoxycholate, 10 mM $Na_4P_2O_7$, 0.5% SDS, and 1% (v/v) Nonidet P40) containing a protease inhibitor cocktail (Complete Mini, Roche). Cells were sonicated and cleared by centrifugation (10,000 g for 10 min, at 4°C), and the whole cell lysate supernatant was collected. Proteins were resolved by SDS/PAGE, transferred on to Protran nitrocellulose membranes (Whatman), and immunoblotted under standard conditions, with primary antibodies as follows: anti-myosin type II MF-20 (Developmental Studies Hybridoma Bank); anti-α-tubulin (Sigma); anti-phospho-Stat3 (Tyr705) D3A7 (Cell Signaling Technology); anti-Stat3 D3Z2G (Cell Signaling); anti-phospho-AKT (Ser473) D9E (Cell Signaling); phospho-Akt (Thr308) (D25E6); anti-AKT (Cell Signaling); anti-phospho-mTOR (Ser2448) D9C2 (Cell

Signaling); anti-mTOR (Cell Signaling); anti-phospho-p70S6 kinase (Thr389) 108D2 (Cell Signaling); anto-phospho-p70 S6 Kinase (Ser371) (Cell Signaling); anti-p70S6 Kinase (Cell Signaling); anti-phospho-p90RSK (Thr359/Ser363) (Cell Signaling); anti-pan p90RSK number 9347 (Cell Signaling); anti-eEF2 (Thr56) (Cell Signaling); and anti-eEF2 (Cell Signaling). Anti-mouse or anti-rabbit immunoglobulin G horseradish peroxidase linked F(ab′)2 secondary antibodies and enhanced chemiluminescence detection kit were from Amersham Biosciences.

2.5. RNA Isolation, RT (Reverse Transcription), and Real Time PCR. C2C12 cells were washed with ice-cold PBS and total RNA was extracted using the TRIzol reagent (Life Technologies). Digestion of contaminating genomic DNA was performed with DNA-free DNase treatment (Ambion). First strand cDNA was generated using a kit with random primers (High Capacity cDNA Reverse Transcription kit, Applied Biosystems) from 1 μg of total RNA. Newly synthesized cDNA was diluted 5-fold in DNA-free water and 5% of this cDNA was used in each real-time PCR assay, using a 7500 Real Time PCR System (Applied Biosystems). The standard curve method was used to calculate the relative mRNA levels for each transcript examined and actin beta (ACTB) was used as a reference to normalize the data. Specific assays used for the Taqman quantification were all from Applied Biosystems: MYOG: Mm00446195_g1; MYOD1: Mm01203489_g1; PAX-7: Mm01354484_m1; SOCS3: Mm00545913_s1; ACTB: Mm00607939_s1.

2.6. Densitometric Analysis and Statistics. Densitometry was performed on scanned immunoblot images (Aida 2.11 software). The gel analysis tool was used to obtain the absolute intensity (AI) for each experimental band and corresponding control band. Relative intensity (RI) for each experimental band was calculated by normalizing the experimental AI to the corresponding control AI. In both densitometric analysis and real-time PCR analysis, the software GraphPad Prism was used to perform statistical analysis with Student's t-test. Results are expressed as means ± s.e.; P values <0.05 were considered statistically significant.

3. Results

3.1. C-26 Adenocarcinoma Cells Secrete IL-6. Proinflammatory cytokines might impinge muscle differentiation both in vitro and in vivo [6–8]. We measured the concentration of IL-6, IL-10, TNF-α, MCP-1, INF-γ, and IL-12p70 secreted in the conditioned medium (CM) by the murine colon C-26 adenocarcinoma cells [5, 18]. This quantitative analysis revealed that C-26 cells secreted and progressively accumulated, in CM, relatively high concentrations of two cytokines, IL-6 and MCP-1 (Figure 1). The levels of the other cytokines secreted in CM by C-26 cells, such as INF-γ, IL-10, TNF-α, and IL-12, were not significantly different compared with the control medium obtained from the C2C12 myogenic cell line (Figure 1).

3.2. C-26 Conditioned Medium Inhibits C2C12 Myogenic Differentiation. In agreement with previously published experiments [24], we observed that when C-26 CM was added to the standard growth medium, C2C12 myoblasts proliferation was impaired (data not shown). To explore if the factors secreted by C-26 adenocarcinoma cells were able to directly interfere with muscle differentiation, we let proliferate C2C12 myoblasts in the standard growth medium until about 80–90% of cell confluence (subconfluence) and then we shifted the cells to the differentiation medium (DM) containing 10% C-26 CM (Figure 2(a)).

Morphological and immunofluorescence analysis revealed that the presence of C-26 CM in DM induced a significant reduction in the number and size of the myosin positive multinucleated myotubes, compared with control C2C12 myotubes (Figure 2(a)). The altered differentiated muscle phenotype was also confirmed by Western blot analysis, revealing a drastic downmodulation of the sarcomeric myosin heavy chain expression, a specific marker of myogenic differentiation (Figures 2(b) and 2(c)).

Our results demonstrate that factors released in the extracellular medium by C-26 adenocarcinoma cells were able to interfere, in a cell autonomous manner, with C2C12 myogenic differentiation and maturation.

3.3. IL-6 Negatively Regulates C2C12 Myogenic Differentiation. Previous studies have shown that C-26 conditioned medium is a complex mixture of secreted proteins, including cytokines, growth factors, and signaling molecules, some of which can potentially play a role in myogenic program and muscle wasting [5, 24]. In this work, using a proinflammatory cytokine array, we have shown that adenocarcinoma C-26 cells can secrete and progressively accumulate in the CM high concentrations of at least two cytokines, IL-6 and MCP-1 (Figure 1).

We explored if IL-6 can recapitulate the inhibitory effect of C-26 CM on C2C12 myogenic differentiation or whether this inhibition is the sum of the concomitant effects of several tumoral factors secreted by C-26 cells.

To avoid any confounding effect, we induced C2C12 myogenic differentiation in the presence of the differentiation medium (DM) supplemented with recombinant exogenous IL-6 protein (rIL-6). We then analysed the differentiated myotubes by Western blotting and immunofluorescence analysis (Figure 2). We observed that the C2C12 myotubes treated with rIL-6 were shorter and smaller in size (Figure 2(a)) and displayed a significant reduction in the sarcomeric myosin heavy chain expression compared with the myotubes differentiated in the control medium (DM) (Figures 2(b) and 2(c)).

Of note, the inhibitory effects of rIL-6 and C-26 CM on C2C12 myogenic differentiation were similar (Figures 2(a)–2(c)).

We also analyzed, by real-time PCR, the effects of rIL-6 on the expression of Pax-7 and MyoD1, two transcription factors that are expressed prevalently in the proliferating myoblasts and in the early stages of the myocytes differentiation [25], and of myogenin, a molecular marker associated

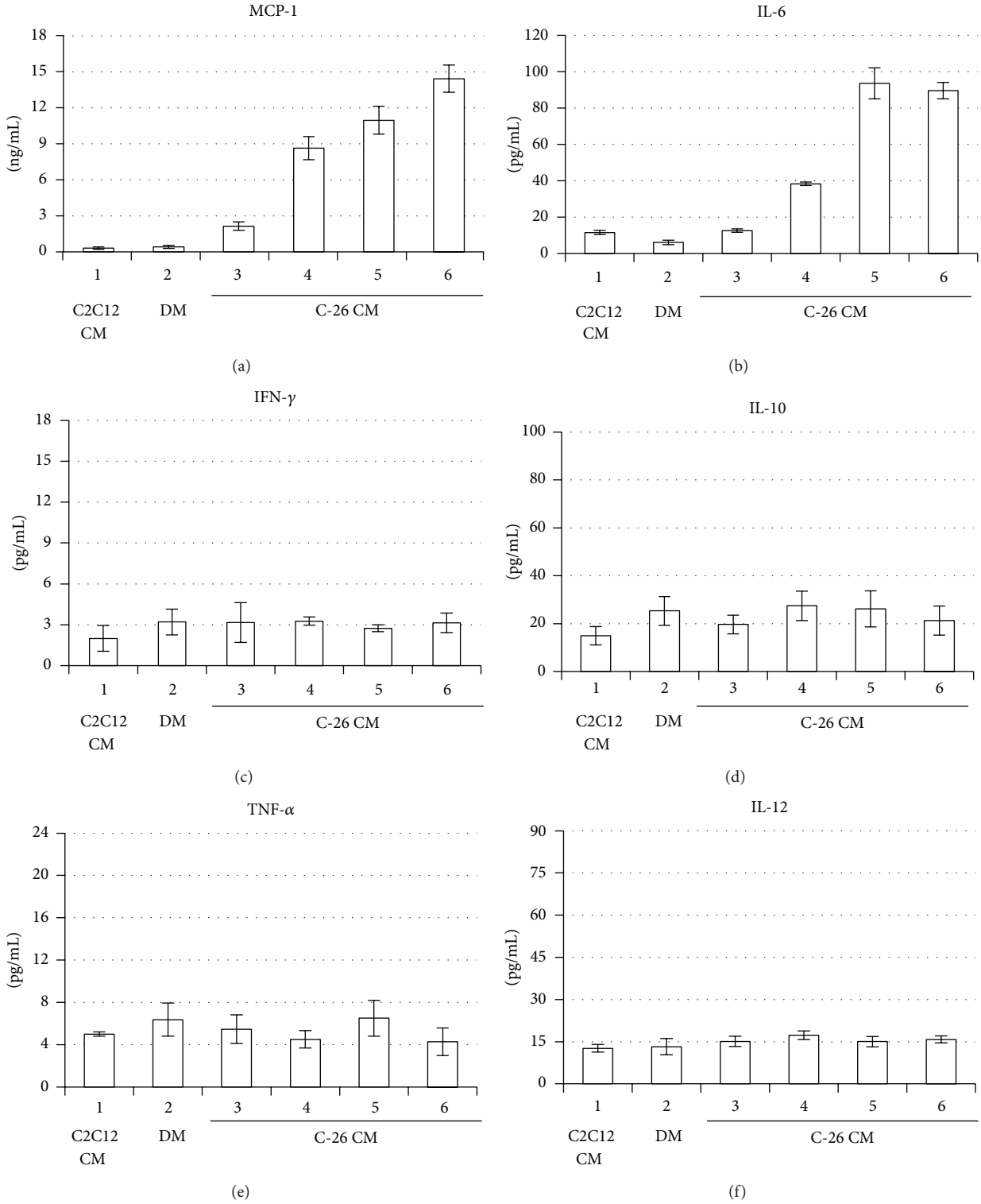


Figure 1: Determination of secreted cytokines in the conditioned medium from C-26 adenocarcinoma cells. (a–f) MCP-1, IL-6, IFN-γ, IL-10, TNF-α, and IL-12p70 protein levels were measured using a cytometric bead assay in which the specific antibodies were conjugated with capture beads (see Section 2). MCP-1 concentration is expressed in ng/mL, whereas the other cytokines concentrations are in pg/mL. 1: control I, conditioned medium (CM) from proliferating C2C12 cells in growth medium (GM); 2: control II, myogenic differentiation medium (DM); 3: CM from proliferating C-26 cells in GM (i.e., exponential phase, with a cell confluence about 50%); 4, 5, and 6: CM from confluent C-26 cells (i.e., stationary phase) with a cell confluence of about 100% for 1, 2, or 3 days, respectively. The experiment indicates means ± standard deviation from three independent determinations.

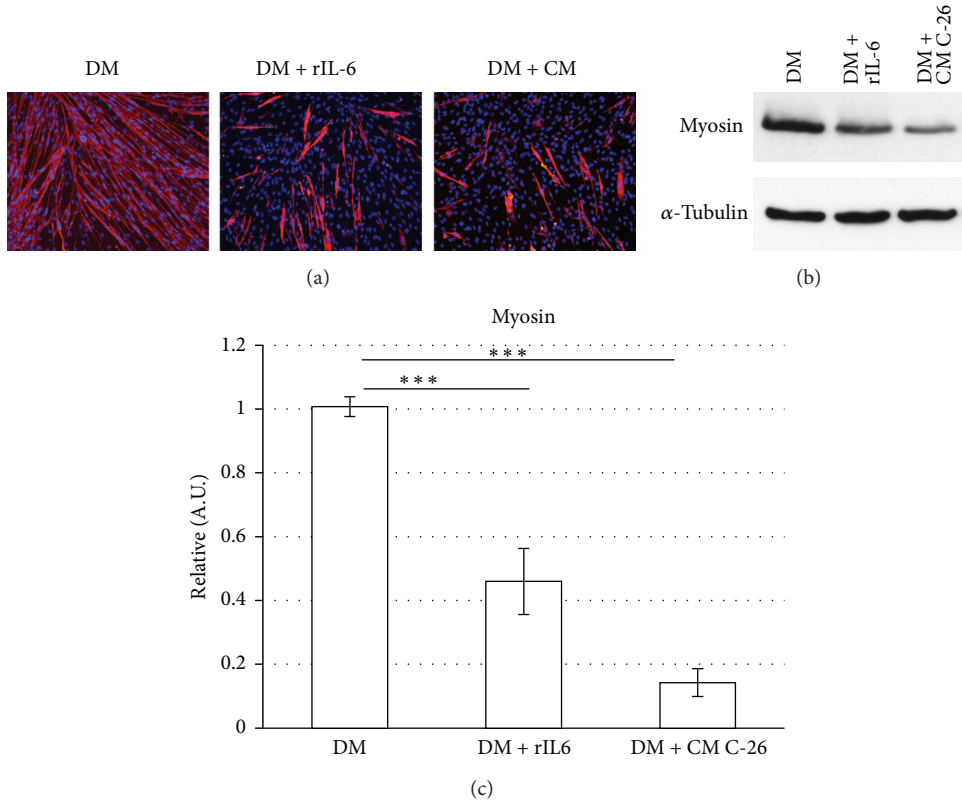


Figure 2: C-26 CM and exogenous IL-6 impair the myogenic differentiation of C2C12 cells. C2C12 myoblasts were cultured in GM until subconfluence and then shifted to differentiation medium (DM), or DM containing 10% C-26 CM, or DM containing 25 ng/mL of recombinant IL-6. (a) After 48 h in the differentiation media, cells were stained using an anti-sarcomeric myosin heavy chain (MF-20) antibody (red) and the Hoechst staining of the DNA (blue) and was imaged by conventional fluorescent microscopy. (b) After 48 h in the differentiation media, cells were harvested and total protein was extracted; cell lysates (70 μg of total protein) were subjected to Western blot analysis with the indicated specific antibodies. The experiment shown is representative of three independent determinations. (c) Densitometric analysis of the relative protein levels (obtained from Western blotting analysis) of sarcomeric myosin heavy chain, represented as arbitrary units (A.U.) and equalized for α-tubulin expression. $^{***}P < 0.005$ (Student's t-test).

with the myocytes' full commitment to differentiation [25] (Figure 3). rIL-6 did not induce any significant change in Pax7 and MyoD expression (Figures 3(a) and 3(b)), whereas we observed a significant downregulation of the myogenin expression compared with controls (Figure 3(c)). On the whole, these data show that exogenous rIL-6 negatively regulates C2C12 myogenic differentiation, without interfering with the early stage of the myogenic program.

3.4. Exogenous IL-6 Activates Stat3-SOCS3 Signaling during C2C12 Myogenic Differentiation. Skeletal muscle expresses the IL-6 receptor (IL6R/CD126) and it is a target of the IL-6 activity [10]. Binding of IL-6 to its receptor initiates specific intracellular events, including phosphorylation and activation of the Janus kinases (JAK) and the subsequent activation of the signal transducer and activator of transcription (Stat) [26, 27]. IL-6-induced Stat signaling also initiates a negative feedback regulation through the increased transcription of the suppressor of cytokine signaling (SOCS) proteins [27].

We analyzed whether rIL-6 treatment activates the canonical signaling through the IL-6 receptor. To this purpose, we induced C2C12 myogenic differentiation in the presence of the differentiation medium (DM) supplemented with rIL-6. Western blot analysis showed that rIL-6 treatment elicited a significant upregulation of the active phosphorylated form (on the Tyr 705 residue) of Stat3 (phospho-Stat3) at the early stages of myogenic differentiation (i.e., day 1; Figure 4(a)), whereas rIL-6 did not induce significant changes in phospho-Stat3 at later stages of the myogenic differentiation (i.e., day 2 and later; Figure 4(a) and data not shown). Real-time PCR analysis showed that the expression of SOCS3 (the suppressor of the IL-6 signaling) was induced by rIL-6 treatments only at the late stage of the myogenic differentiation (i.e., day 2 and later; Figure 4(b) and data not shown).

In conclusion, this experiment shows that in C2C12 cells the exogenous rIL-6 is able to efficiently activate the Stat3-SOCS3 signaling during myogenic differentiation; moreover, the cytokine-inducible positive activation of Stat3 takes place at the early stages of myogenic differentiation, whereas the negative feedback of the signaling pathway (promoted by the Stat signaling itself) is activated at later differentiation stages, through the transcriptional induction of SOCS3.

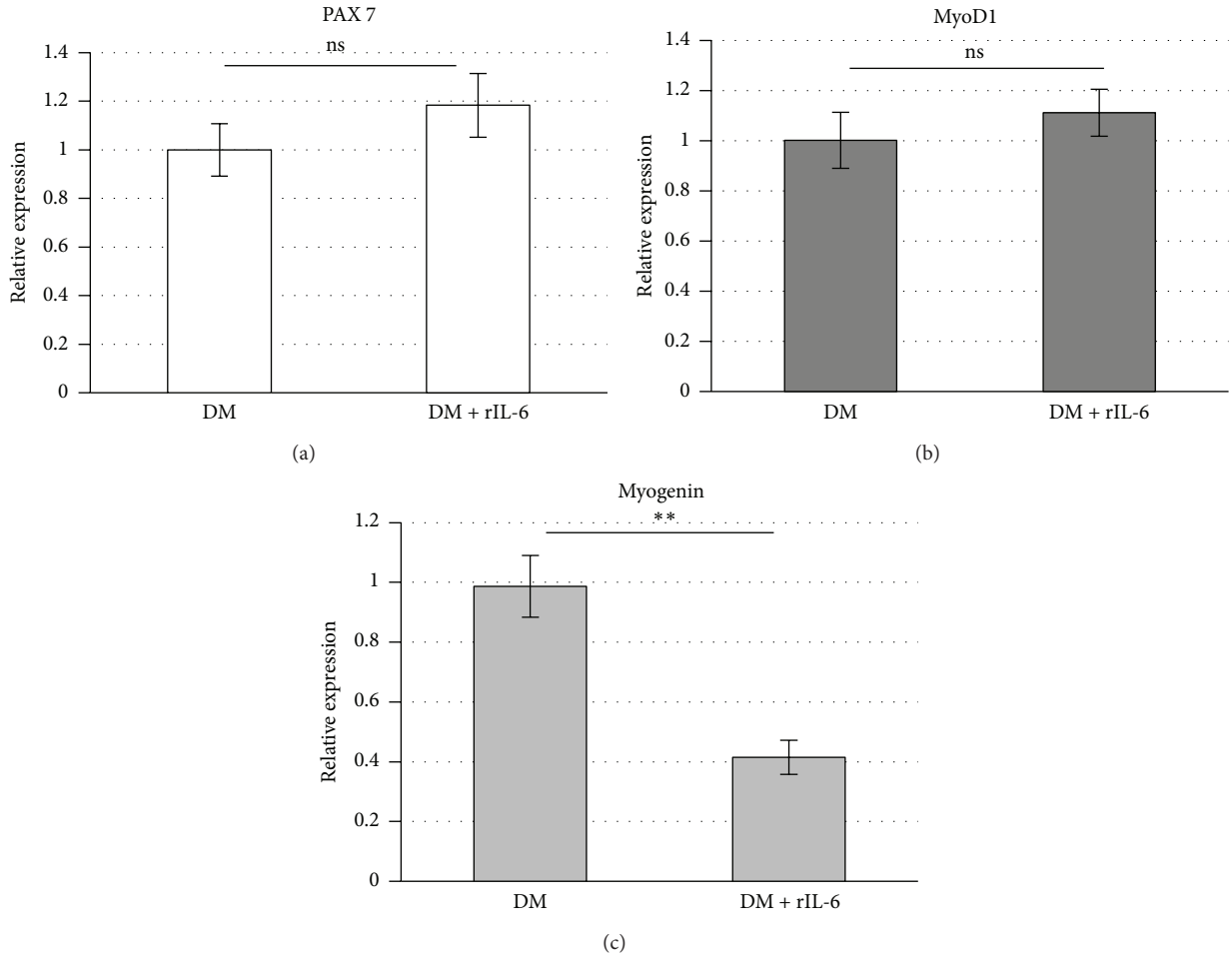


Figure 3: Exogenous IL-6 impairs the expression of myogenin, without affecting the expression of Pax-7 and MyoD. C2C12 myoblasts were cultured in GM until subconfluence and then shifted to DM or DM containing 25 ng/mL of recombinant IL-6. After 48 h in the differentiation media, cells were harvested, total RNA was extracted, and first-strand cDNA was generated from 1 μg of total RNA to perform real-time PCR using specific Taqman probes. Expression data were normalized using actin beta (ACTB) as housekeeping gene. Results are expressed as means ± standard deviations. $^{**}P < 0.005$; n.s., not significant (Student's t-test).

3.5. Exogenous IL-6 Perturbs mTOR and p70S6K Activities, without Affecting AKT Activity, during C2C12 Myogenic Differentiation. AKT inhibition can substantially contribute to impinge muscle differentiation and muscle atrophy [19, 20, 28]. AKT plays a central role in the control of both muscle protein synthesis, via mTOR, and protein degradation, via the transcription factors of the FoxO family [19, 20].

Thus, we analyzed if exogenous rIL-6 could perturb AKT signaling pathways during C2C12 myogenic differentiation. We induced C2C12 differentiation in the presence of DM supplemented with rIL-6 and then analyzed the differentiated myotubes by Western blotting using specific antibodies (Figure 5). In the C2C12 myotubes differentiated in the presence of rIL-6 we did not observe any significant change in the levels of both the total AKT protein and the phosphorylated/active forms of AKT, namely, phospho-AKT Thr308 and phospho-AKT Ser473 (Figures 5(a)–5(d)). However, rIL-6 induced a significant reduction in the phosphorylated/active forms of mTOR (phospho-Ser2448) and p70S6K (phospho-Thr389 and phospho-Ser371) (Figures 5(e)–5(i)). The 2 kinases mTOR and p70S6K are among the crucial downstream effectors of the AKT pathway and positively control protein synthesis and muscle differentiation [29].

In conclusion, this experiment shows that, during C2C12 myogenic differentiation, AKT does not sustain alone the activity of mTOR and p70S6K1. We suggest that an ancillary pathway should contribute to sustain the activity of mTOR and p70S6K during C2C12 myogenic differentiation and when this ancillary pathway is inhibited, by exogenous rIL-6, the activation of mTOR and p70S6K is perturbed and the myotubes formation is impaired (see Figures 2 and 5).

3.6. Exogenous IL-6 Affects the Phosphorylation Levels of p90RSK and eEF2 during C2C12 Myogenic Differentiation. It has been demonstrated that, similar to AKT, signaling generated from p90RSK plays a positive role in the regulation of protein synthesis during myogenic differentiation and maturation [21, 22]. Active p90RSK increases the levels of

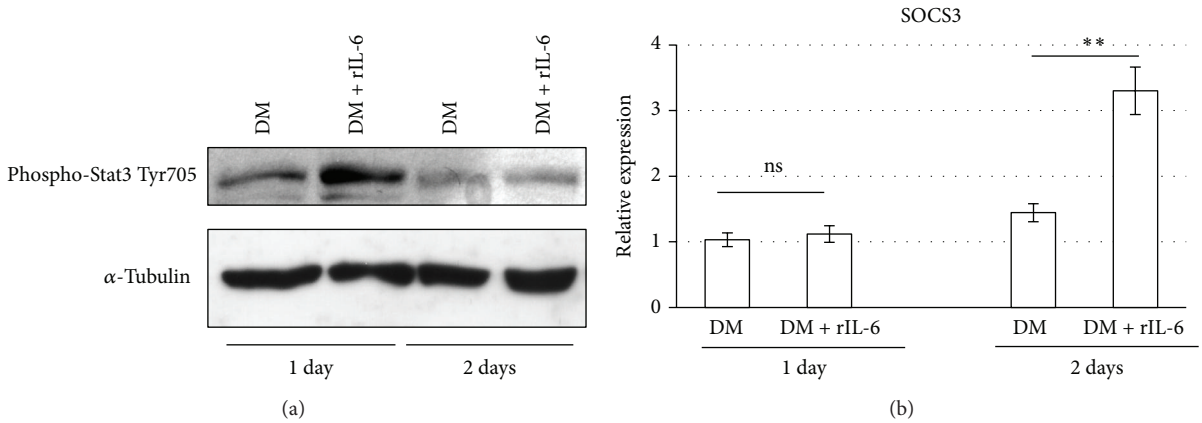


Figure 4: Exogenous IL-6 activates Stat3-SOCS3 signaling during myogenic differentiation. C2C12 myoblasts were cultured in GM until subconfluence and then shifted to DM or DM containing 25 ng/mL of recombinant IL-6. After 24 h or 48 h in the differentiation media, cells were harvested and total protein was extracted, or total RNA was extracted and first-strand cDNA was generated from 1 μg of total RNA. (a) Cell lysates (70 μg of total proteins) were subjected to Western blotting analysis with the indicated specific antibodies. The experiment shown is representative of three independent determinations. (b) Real-time PCR was performed on cDNA, using the indicated specific Taqman probes. Expression data were normalized using actin beta (ACTB) as housekeeping gene. The experiment shows means ± standard deviation of three independent determinations. $^{**}P < 0.005$; n.s., not significant (Student's t test).

the dephosphorylated (active) form of eEF2, which catalyzes the translocation of peptidyl-tRNA from the A site to the P site on the ribosome [21]. Moreover, p90RSK can promote mTOR and p70S6K1 activation independently from AKT (Figure 6(d) and [23]). Thus, we explored if IL-6 could perturb p90RSK signaling pathways during C2C12 myogenic differentiation (Figure 6). We induced C2C12 differentiation in the presence of DM supplemented with rIL-6. IL-6 was able to induce a negative regulation of p90RSK signaling in the myogenic cells. As shown in Figures 6(a) and 6(b), p90RSK phosphorylation/activation was significantly downregulated in C2C12 differentiating cells treated with rIL6, and the reduced p90RSK active form was associated with a significant increase in the levels of the eEF2 phosphorylation (Figures 6(a)–6(c)), which is involved in the downmodulation of the translocation step in the protein synthesis [21].

In summary, in this study we suggest that, during C2C12 myogenic differentiation, exogenous rIL-6 can induce a depression of the protein synthesis in the myotubes, via a concomitant downmodulation of the p90RSK/eEF2 axis and of the mTOR/p70S6K, without apparently affecting the AKT activity.

4. Discussion

There is increasing evidence demonstrating the critical role of IL-6 in muscle homeostasis, regeneration, and differentiation [9]. Mouse models in which IL-6 has been locally chronically elevated in skeletal muscle, through transgenic overexpression [14], in vivo electroporation [15], or being infused directly into muscle [16], induced muscle atrophy. Finally, it has been shown that administration of IL-6 neutralizing antibodies or IL-6 inhibitors can reduce muscle wasting in tumor-bearing mice [5, 30].

In this study, we have shown that the murine colon C-26 adenocarcinoma cell line (C-26) secreted in the extracellular medium significant concentrations of two cytokines, IL-6 and MCP-1, whereas we could not detect any significant accumulation of other relevant inflammatory cytokines tested, namely, INF-γ, IL-10, TNF-α, and IL-12 (Figure 1).

The accumulation of the proinflammatory cytokine IL-6 in the extracellular medium of the C-26 cancer cells was not unexpected. Strassmann et al., in fact, reported that some clones of C-26 adenocarcinoma cells can secrete IL-6 and linked this cytokine to the development of a severe cachexia associated with muscle wasting [5]. It has been shown that mice implanted with the C-26 cells have an increased circulating IL-6 concentration, which coincides with muscle wasting [5].

In this study, we found that, in addition to IL-6, C-26 cells also secreted and accumulated in the extracellular medium the monocyte chemotactic protein-1 (MCP-1). MCP-1 is a chemokine and member of the small inducible cytokine family, playing a crucial role in the recruitment of monocytes and T lymphocytes into tissues [31]. In nonpathological conditions MCP-1 is expressed by adipocytes and by a number of other cell types, including smooth muscle and endothelial cells exposed to inflammatory stimuli [31]. MCP-1 has also been shown to play a prominent role as an inducer of insulin resistance in human skeletal muscle cells [32]. This latter role of MCP-1 may link directly this cytokine to the downmodulation of protein synthesis and/or to the increase in protein degradation and, in this way, to cancer cachexia. In this paper, we did not tackle the possible implication of MCP-1 on myogenic program, which will be addressed in a further dedicated investigation.

In our study, we aimed to explore the specific effect of IL-6 on the myogenic program, with the goal to demonstrate whether IL-6 was able to recapitulate the inhibitory effect

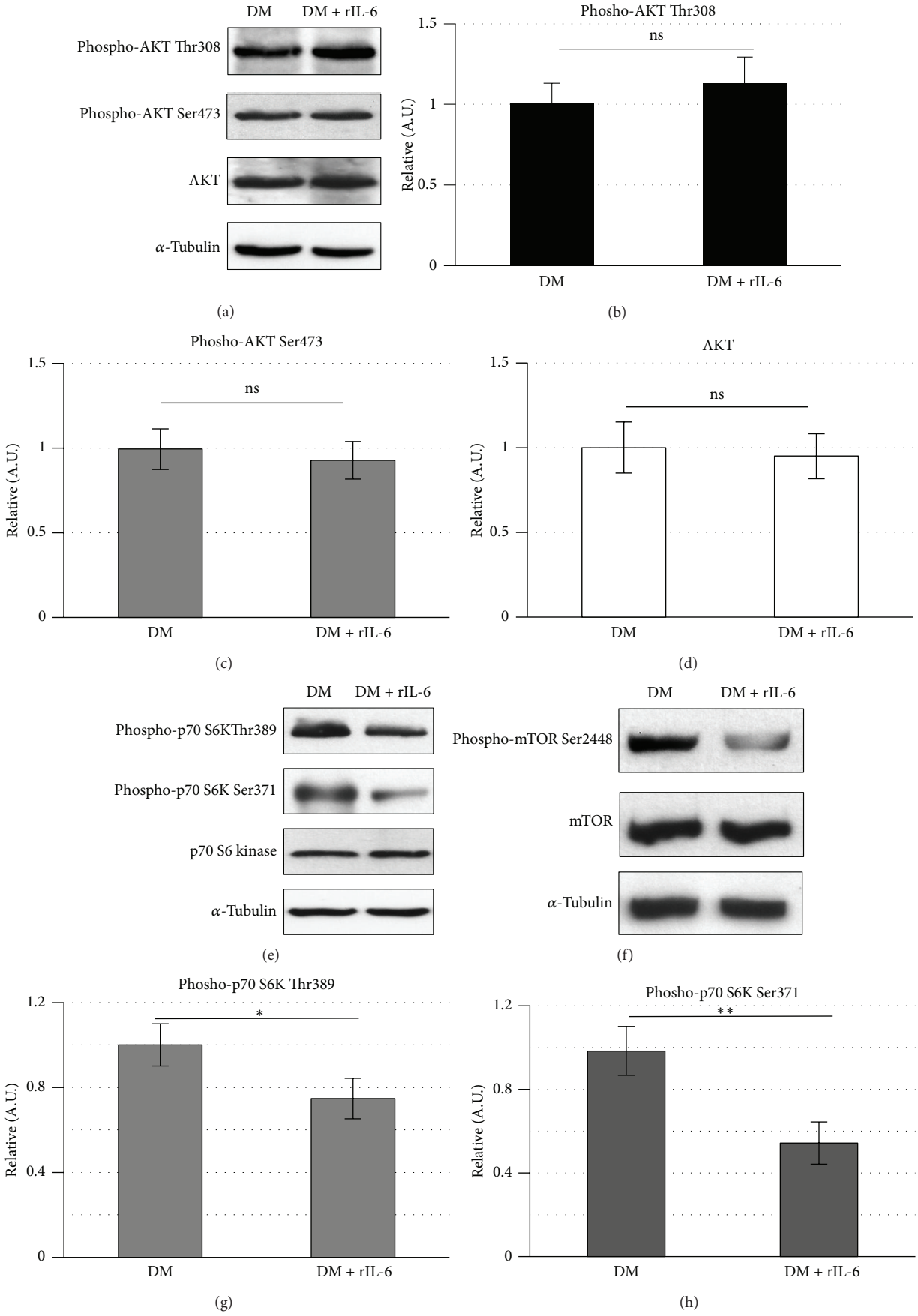


Figure 5: Continued.

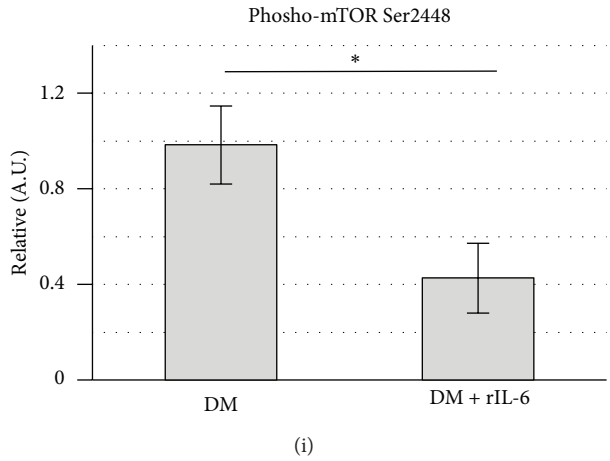


Figure 5: IL-6 perturbs mTOR and p70S6K activities, without affecting AKT activity. C2C12 myoblasts were cultured in GM until subconfluence and then shifted to DM or DM containing 25 ng/mL of recombinant IL-6. (a, e, and f) After 48 h in the DM, cells were harvested and total protein was extracted; cell lysates (70 μg of total proteins) were subjected to Western blotting analysis with the indicated specific antibodies. Each experiment shown is representative of three independent determinations. (b, c, d, g, h, and i) Densitometric analysis of the relative protein levels (obtained from Western blotting analysis), represented as arbitrary units (A.U.) and equalized for total AKT expression (b and c), α-tubulin expression (d), total p70S6K expression (g and h), or total mTOR expression (i). The experiments show means ± standard deviation of three independent determinations. n.s., not significant; $^{*}P < 0.05$; $^{**}P < 0.005$ (Student's t test).

of C-26 CM on C2C12 myogenic differentiation or whether this inhibition required the sum of the concomitant effects of several factors secreted by C-26 cells. Thus, to avoid any confounding result, we restricted the analysis to the proinflammatory cytokine IL-6. We added recombinant IL-6 (rIL-6) in the differentiation medium (DM) and we found that this cytokine is able to recapitulate the key inhibitory effects of C-26 CM on C2C12 myogenic differentiation. The presence of exogenous recombinant IL-6 in the DM induced a significant downregulation in the expression of the late markers of myogenic differentiation, like myogenin and the sarcomeric myosin heavy chain, whereas it did not induce any significant change in the expression of transcription factors that are expressed prevalently in the proliferating myoblasts and at the early stages of the myocytes differentiation [25] (Figure 2). This suggests that rIL-6 impinges muscle differentiation without hampering the early stage of the myogenic program.

We have also shown that the negative effects of rIL-6 on muscle differentiation are apparently mediated by the canonical activation of the Stat-SOCS signaling pathway (Figure 4). Our results are in accordance with a context in which IL-6 can play a prominent role in the cancer-induced muscle wasting [2, 4, 5, 11]. However, other reports have shown that in myogenic cells the autocrine production of IL-6 can also induce Stat3 activation but promotes the myogenic differentiation [11, 12]. It has been shown that IL-6 mRNA knockdown reduces muscle-specific gene expression in cultured C2C12 myoblasts [11], suggesting a potential myogenic role for the cytokine. Moreover, Serrano et al. identified IL-6 as an essential regulator of satellite cell-mediated hypertrophic muscle growth [13]. This apparent paradox can be justified considering the levels of expression of IL-6, its spatial and temporal interaction with the target tissue, and the length of time in which the circulating IL-6 is actually elevated, that is, a chronic response versus a somewhat brief increase that returns to baseline [33].

Skeletal muscle cells are at the same time a source and a target of IL-6 [10]. It has been shown that, with exercise and under physiological conditions, skeletal muscle-produced IL-6 can be elevated in the circulation for several hours and that the autocrine effects of IL-6 contribute to activation of the Stat3-SOCS3 signaling and to the positive regulation of the myogenic differentiation [10, 33]. By contrast, under pathologic conditions, including cancer cachexia, muscular dystrophy, and chronic inflammatory diseases, there is a chronic (i.e., long lasting) elevation of the circulating IL-6; although Stat3-SOCS3 signaling is also activated in the target skeletal muscle tissue under these pathologic conditions, the autocrine loop of regulation in the action of the cytokine is totally lost. It is possible that different intracellular signaling pathways are activated and this may explain why IL-6 can function both as positive and negative regulator of the myogenesis in physiological or in pathological conditions.

In skeletal muscle, AKT plays a very central role in the control of both muscle protein synthesis, via mTOR, and protein degradation, via the transcription factors of the FoxO family [19, 29]. However, in this study we found that rIL-6 did not directly affect AKT expression and activity during C2C12 myogenic differentiation (Figure 5).

Instead, we found that rIL-6 induced the concomitant downmodulation of two signaling axes which are downstream to AKT and are important for the regulation of the protein synthesis, namely, p90RSK/eEF2 and mTOR/p70S6K (see Figure 5 and [19, 21, 23, 29]).

In muscle cells, activated p90RSK phosphorylates and inactivates the elongation factor 2 kinase (eEF2K); inhibition

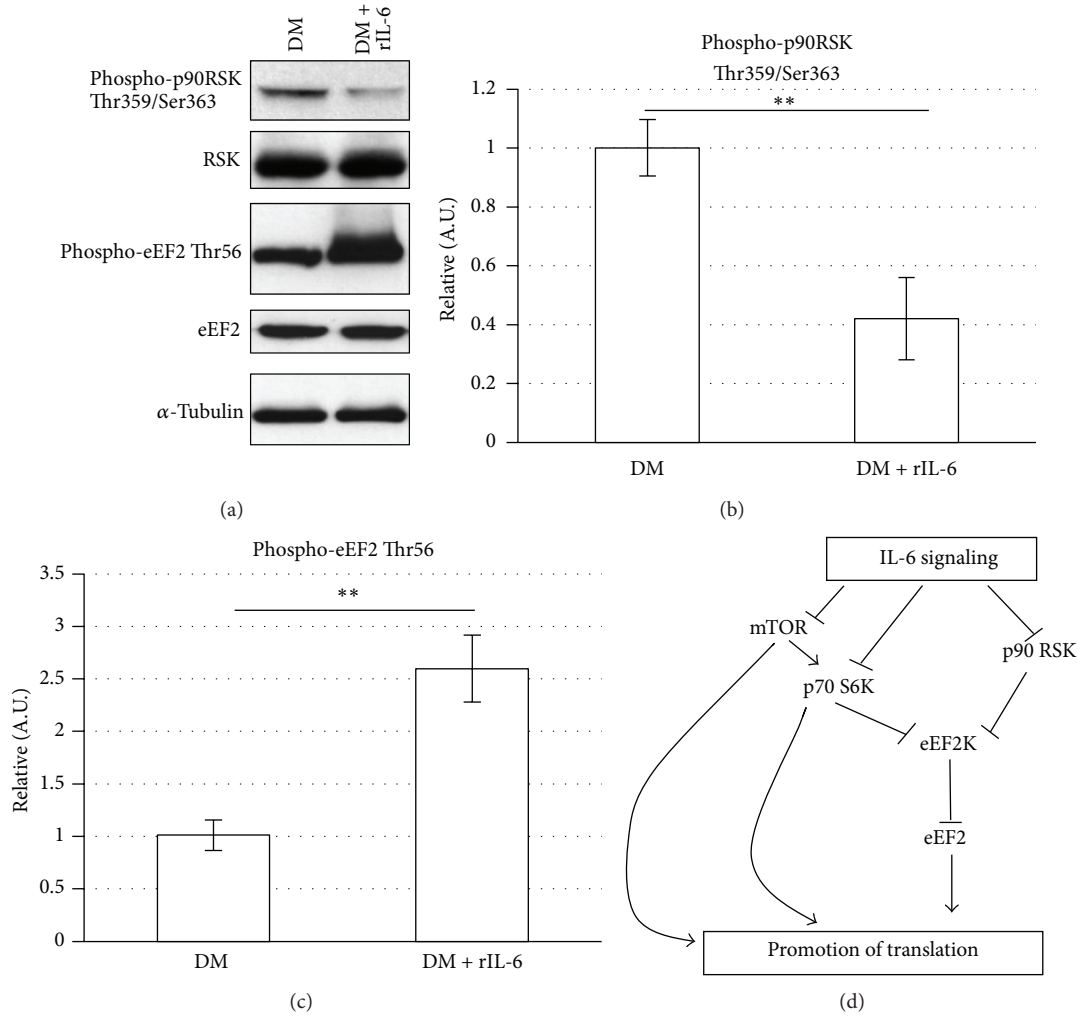


Figure 6: IL-6 affects the phosphorylation levels of p90RSK and eEF2. C2C12 myoblasts were cultured in GM until subconfluence and then shifted to DM or DM containing 25 ng/mL of recombinant IL-6. (a and c) After 48 h in the DM, C2C12 cells were harvested and total protein was extracted; cell lysates (70 μg of total proteins) were subjected to Western blotting analysis with the indicated specific antibodies. Each experiment shown is representative of three independent determinations. (b and d) Densitometric analysis of the relative protein levels (obtained from Western blotting analysis), represented as arbitrary units (A.U.) and equalized for total RSK (b) or total eEF2 (c). The experiments show means ± standard deviation of three independent determinations. $^{**}P < 0.005$ (Student's t-test). (e) A schematic model of the signaling connections was highlighted and discussed in this study.

of eEF2K activity results in a decrease in the inhibitory phosphorylation of eEF2 [21]. Indeed, only the dephosphorylated form of eEF2 is active and can catalyze the translocation of the peptidyl-tRNA from the A site to the P site on the ribosome and finally promote the protein synthesis [21] (see Figure 6(d)).

Activated p90RSK1 can also promote the protein synthesis acting on mTOR/p70S6K1 pathway independently of AKT activation [23] (Figure 6(d)). In fact, p90RSK1 has been found to interact with and phosphorylate the protein tuberin in a specific regulatory site and this phosphorylation inhibits the function of the tuberin-hamartin complex, resulting in increased mTOR signalling to p70S6K1 [9]. Finally, eEF2K activity can also be regulated by p70S6K; this latter kinase can in fact phosphorylate eEF2K and, as p90RSK1, can inhibit its activity [21].

It is evident that destabilization of this very intricate network of signaling pathways by the exogenous IL-6 (i.e., by the chronic elevation of circulating IL-6) can contribute to the decrease of the muscle protein synthesis and the promotion of muscle wasting (Figure 6(d)).

Our findings thus identify the p90RSK/eEF2 and mTOR/p70S6K axes as new potential targets of IL-6 activity on the myogenic program.

5. Conclusions

This study clarified the possible role of the IL-6 in the induction of skeletal muscle wasting. We show that rIL-6 impairs C2C12 myogenic differentiation, activates the Stat3-SOCS3 signaling pathway, and induces the downmodulation of p90RSK/eEF2 and mTOR/p70S6K axes, which are important

to stimulate the protein synthesis and in the maintenance of the differentiated myogenic phenotype. Exogenous rIL-6 does not affect the expression levels and activation of AKT, a kinase essential to promote protein synthesis and cell survival and to block protein degradation in skeletal muscle cells.

Moreover, in this study we discuss how the pleiotropic cytokine IL-6 can contribute to the positive regulation of the myogenic differentiation under physiological conditions and to the negative regulation of the myogenic phenotype under some pathological circumstances.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Michele Pelosi and Manuela De Rossi equally contributed to this paper.

Acknowledgments

This work was supported by PRIN, Telethon, ASI, AFM, and Regione Lazio.

References

[1] C. H. Clegg, T. A. Linkhart, B. B. Olwin, and S. D. Hauschka, "Growth factor control of skeletal muscle differentiation: commitment to terminal differentiation occurs in G1 phase and is repressed by fibroblast growth factor," *Journal of Cell Biology*, vol. 105, no. 2, pp. 949–956, 1987.

[2] N. E. Olson, E. Sternberg, J. S. Hu, G. Spizz, and C. Wilcox, "Regulation of myogenic differentiation by type β transforming growth factor," *Journal of Cell Biology*, vol. 103, no. 5, pp. 1799–1805, 1986.

[3] A. Musarò and N. Rosenthal, "Maturation of the myogenic program is induced by postmitotic expression of insulin-like growth factor I," *Molecular and Cellular Biology*, vol. 19, no. 4, pp. 3115–3124, 1999.

[4] A. Musarò, K. J. A. McCullagh, F. J. Naya, E. N. Olson, and N. Rosenthal, "IGF-1 induces skeletal myocyte hypertrophy through calcineurin in association with GATA-2 and NF-ATc1," *Nature*, vol. 400, no. 6744, pp. 581–585, 1999.

[5] G. Strassmann, M. Fong, J. S. Kenney, and C. O. Jacob, "Evidence for the involvement of interleukin 6 in experimental cancer cachexia," *The Journal of Clinical Investigation*, vol. 89, no. 5, pp. 1681–1684, 1992.

[6] S. C. Miller, H. Ito, H. M. Blau, and F. M. Torti, "Tumor necrosis factor inhibits human myogenesis in vitro," *Molecular and Cellular Biology*, vol. 8, no. 6, pp. 2295–2301, 1988.

[7] D. Coletti, E. Yang, G. Marazzi, and D. Sassoon, "TNFα inhibits skeletal myogenesis through a PW1-dependent pathway by recruitment of caspase pathways," *EMBO Journal*, vol. 21, no. 4, pp. 631–642, 2002.

[8] D. C. Guttridge, M. W. Mayo, L. V. Madrid, C.-Y. Wang, and J. Baldwin A.S., "NF-κB-induced loss of MyoD messenger RNA: possible role in muscle decay and cachexia," *Science*, vol. 289, no. 5488, pp. 2363–2365, 2000.

[9] P. Muñoz-Cánoves, C. Scheele, B. K. Pedersen, and A. L. Serrano, "Interleukin-6 myokine signaling in skeletal muscle: a double-edged sword?" *FEBS Journal*, vol. 280, no. 17, pp. 4131–4148, 2013.

[10] B. K. Pedersen and M. A. Febbraio, "Muscle as an endocrine organ: focus on muscle-derived interleukin-6," *Physiological Reviews*, vol. 88, no. 4, pp. 1379–1406, 2008.

[11] B. Baeza-Raja and P. Muñoz-Cánoves, "p38 MAPK-induced nuclear factor-kappaB activity is required for skeletal muscle differentiation: role of interleukin-6," *Molecular Biology of the Cell*, vol. 15, no. 4, pp. 2013–2026, 2004.

[12] M. Hoene, H. Runge, H. U. Häring, E. D. Schleicher, and C. Weigert, "Interleukin-6 promotes myogenic differentiation of mouse skeletal muscle cells: role of the STAT3 pathway," *American Journal of Physiology. Cell Physiology*, vol. 304, no. 2, pp. C128–C136, 2013.

[13] A. L. Serrano, B. Baeza-Raja, E. Perdiguero, M. Jardí, and P. Muñoz-Cánoves, "Interleukin-6 is an essential regulator of satellite cell-mediated skeletal muscle hypertrophy," *Cell Metabolism*, vol. 7, no. 1, pp. 33–44, 2008.

[14] F. De Benedetti, C. Meazza, and A. Martini, "Role of interleukin-6 in growth failure: an animal model," *Hormone Research*, vol. 58, supplement 1, pp. 24–27, 2002.

[15] S. Franckhauser, I. Elias, V. Rotter Sopasakis et al., "Overexpression of Il6 leads to hyperinsulinaemia, liver inflammation and reduced body weight in mice," *Diabetologia*, vol. 51, no. 7, pp. 1306–1316, 2008.

[16] F. Haddad, F. Zaldivar, D. M. Cooper, and G. R. Adams, "IL-6-induced skeletal muscle atrophy," *Journal of Applied Physiology*, vol. 98, no. 3, pp. 911–917, 2005.

[17] R. L. Perry and M. A. Rudnick, "Molecular mechanisms regulating myogenic determination and differentiation," *Frontiers of Bioscience*, vol. 1, no. 5, pp. D750–D767, 2000.

[18] Y. Tanaka, H. Eda, T. Tanaka et al., "Experimental cancer cachexia induced by transplantable colon 26 adenocarcinoma in mice," *Cancer Research*, vol. 50, no. 8, pp. 2290–2295, 1990.

[19] P. Bonaldo and M. Sandri, "Cellular and molecular mechanisms of muscle atrophy," *Disease Models & Mechanisms*, vol. 6, no. 1, pp. 25–39, 2013.

[20] B. M. Scicchitano, E. Rizzuto, and A. Musarò, "Counteracting muscle wasting in aging and neuromuscular diseases: the critical role of IGF-1," *Aging*, vol. 1, no. 5, pp. 451–457, 2009.

[21] X. Wang, W. Li, M. Williams, N. Terada, D. R. Alessi, and C. G. Proud, "Regulation of elongation factor 2 kinase by p90 (RSK1) and p70 S6 kinase," *The EMBO Journal*, vol. 20, no. 16, pp. 4370–4379, 2001.

[22] M. Pelosi, F. Marampon, B. M. Zani et al., "ROCK2 and its alternatively spliced isoform ROCK2m positively control the maturation of the myogenic program," *Molecular and Cellular Biology*, vol. 27, no. 17, pp. 6163–6176, 2007.

[23] P. P. Roux, B. A. Ballif, R. Anjum, S. P. Gygi, and J. Blenis, "Tumor-promoting phorbol esters and activated Ras inactivate the tuberous sclerosis tumor suppressor complex via p90 ribosomal S6 kinase," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 37, pp. 13489–13494, 2004.

[24] S. Lokireddy, I. W. Wijesoma, S. Bonala et al., "Myostatin is a novel tumoral factor that induces cancer cachexia," *The Biochemical Journal*, vol. 446, no. 1, pp. 23–36, 2012.

[25] C. F. Bentzinger, Y. X. Wang, N. A. Dumont, and M. A. Rudnicki, "Cellular dynamics in the muscle satellite cell niche," *EMBO Reports*, vol. 14, no. 12, pp. 1062–1072, 2013.

[26] M. Mihara, M. Hashizume, H. Yoshida, M. Suzuki, and M. Shiina, "IL-6/IL-6 receptor system and its role in physiological and pathological conditions," *Clinical Science*, vol. 122, no. 4, pp. 143–159, 2012.

[27] P. C. Heinrich, I. Behrmann, S. Haan, H. M. Hermanns, G. Müller-Newen, and F. Schaper, "Principles of interleukin (IL)-6-type cytokine signalling and its regulation," *The Biochemical Journal*, vol. 374, no. 1, pp. 1–20, 2003.

[28] X.-D. Peng, P.-Z. Xu, M.-L. Chen et al., "Dwarfism, impaired skin development, skeletal muscle atrophy, delayed bone development, and impeded adipogenesis in mice lacking Akt1 and Akt2," *Genes and Development*, vol. 17, no. 11, pp. 1352–1365, 2003.

[29] M. Sandri, "Signaling in muscle atrophy and hypertrophy," *Physiology*, vol. 23, no. 3, pp. 160–170, 2008.

[30] M. H. Zaki, J. A. Nemeth, and M. Trikha, "CNTO 328, a monoclonal antibody to IL-6, inhibits human tumor-induced cachexia in nude mice," *International Journal of Cancer*, vol. 111, no. 4, pp. 592–595, 2004.

[31] S. L. Deshmane, S. Kremlev, S. Amini, and B. E. Sawaya, "Monocyte chemoattractant protein-1 (MCP-1): an overview," *Journal of Interferon and Cytokine Research*, vol. 29, no. 6, pp. 313–325, 2009.

[32] H. Sell, D. Dietze-Schroeder, U. Kaiser, and J. Eckel, "Monocyte chemotactic protein-1 is a potential player in the negative cross-talk between adipose tissue and skeletal muscle," *Endocrinology*, vol. 147, no. 5, pp. 2458–2467, 2006.

[33] J. A. Carson and K. A. Baltgalvis, "Interleukin 6 as a key regulator of muscle mass during cachexia," *Exercise and Sport Sciences Reviews*, vol. 38, no. 4, pp. 168–176, 2010.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 235426, 14 pages
http://dx.doi.org/10.1155/2014/235426

Research Article

Local Overexpression of V1a-Vasopressin Receptor Enhances Regeneration in Tumor Necrosis Factor-Induced Muscle Atrophy

Alessandra Costa,[1] Angelica Toschi,[1] Ivana Murfuni,[1] Laura Pelosi,[1] Gigliola Sica,[2] Sergio Adamo,[1] and Bianca Maria Scicchitano[1,2]

[1] *DAHFMO Unit of Histology and Medical Embryology, Interuniversity Institute of Myology, Sapienza University of Rome, Via A. Scarpa 16, 00161 Rome, Italy*
[2] *Institute of Histology and Embryology, Catholic University School of Medicine, L.go F. Vito, 1, 00168 Rome, Italy*

Correspondence should be addressed to Sergio Adamo; sergio.adamo@uniroma1.it

Received 27 January 2014; Revised 22 April 2014; Accepted 23 April 2014; Published 20 May 2014

Academic Editor: Marina Bouché

Skeletal muscle atrophy occurs during disuse and aging, or as a consequence of chronic diseases such as cancer and diabetes. It is characterized by progressive loss of muscle tissue due to hypotrophic changes, degeneration, and an inability of the regeneration machinery to replace damaged myofibers. Tumor necrosis factor (TNF) is a proinflammatory cytokine known to mediate muscle atrophy in many chronic diseases and to inhibit skeletal muscle regeneration. In this study, we investigated the role of Arg-vasopressin-(AVP-)dependent pathways in muscles in which atrophy was induced by local overexpression of TNF. AVP is a potent myogenesis-promoting factor and is able to enhance skeletal muscle regeneration by stimulating Ca^{2+}/calmodulin-dependent kinase and calcineurin signaling. We performed morphological and molecular analyses and demonstrated that local over-expression of the AVP receptor V1a enhances regeneration of atrophic muscle. By upregulating the regeneration/differentiation markers, modulating the inflammatory response, and attenuating fibrogenesis, the stimulation of AVP-dependent pathways creates a favourable environment for efficient and sustained muscle regeneration and repair even in the presence of elevated levels of TNF. This study highlights a novel *in vivo* role for AVP-dependent pathways, which may represent an interesting strategy to counteract muscle decline in aging or in muscular pathologies.

1. Introduction

Skeletal muscle is a dynamic tissue capable of extensive regeneration in response to injury. Nevertheless, regeneration may be hindered in the case of aging, extended injury, or pathological conditions, leading to functional impairment. Although the general mechanisms underlying muscle regeneration have been identified, little is known about the factors limiting efficient repair in pathological muscle. Reduction in the number of satellite cells, poor recruitment of circulating stem cells within the damaged area, chronic inflammation, and formation of fibrotic tissue represent important factors contributing to limited or impaired regeneration. Inflammation is an important phase in the muscle regeneration process [1, 2]. Cytokines expressed during the early phase of inflammatory responses, such as interferon-γ (IFN-γ), interleukin-1β (IL-1β), and TNF, drive the differentiation and activation of macrophages towards the M1 phenotype. These classically activated M1 macrophages represent a proinflammatory population of cells capable of amplifying and perpetuating the inflammatory response [1]. After M1 macrophages reach their peak concentration, they are replaced by a population of alternatively activated M2 macrophages, which are activated by anti-inflammatory cytokines such as interleukin-4 (IL-4), interleukin-10 (IL-10), express CD-163 [1, 3, 4], and attenuate the inflammatory response and promote tissue repair. Perturbations in the spatial distribution of inflammatory cells, changes in the type and magnitude of the inflammatory

cell infiltrate, and disrupted temporal sequence, result in a persistent rather than resolved inflammatory phase [5] and functional impairment of skeletal muscle [6].

The nuclear-factor-kappa B (NF-κB) transcription factor is one of the most important molecules, which has been linked to the loss of skeletal muscle mass in various physiopathological conditions. Activation of NF-κB in skeletal muscle leads to degradation of muscle specific proteins, inflammation, and fibrosis and blocks myofiber regeneration after injury/atrophy [7, 8]. Interestingly, NF-κB regulates the expression of a number of inflammatory molecules including proinflammatory cytokines such as TNF, which is also a potent activator of NF-κB, thus establishing a positive feedback loop leading to muscular abnormalities [9]. Although the autoactivation of NF-κB requires proteolytic processing of NF-κB and IκB family of proteins by the ubiquitin-proteasome system [9, 10], NF-κB can induce the expression of FoxO transcription factors by modulating the Akt signaling pathways [11]. Nuclear translocation and activation of FoxO transcription factors results in the upregulation of atrogin-1/MAFbx and MuRF1, thus promoting atrophy and muscle loss [12–14].

In this study, we examined the effects of local V1a-vasopressin receptor overexpression on TNF-induced muscle atrophy. The neurohypophyseal nonapeptide Arg^8-vasopressin (AVP), oxytocin (OT), and related peptides constitute a family of positive regulators of terminal differentiation in myogenic cell lines (L5 and L6) and primary satellite cells [15–17]. V1aR is the only vasopressin receptor expressed in skeletal muscle [18–20]. Previously, we demonstrated that V1aR expression is modulated during skeletal muscle regeneration, and that AVP-V1aR signaling is a powerful enhancer of muscle regeneration through mechanisms involving calcineurin A (CnA), GATA2, MEF2, and IL-4 [21]. There is now extensive evidence showing that the AVP system is impaired in several neuromuscular diseases, such as amyotrophic lateral sclerosis and multiple sclerosis [22, 23], suggesting that AVP may act as a physiologic factor in skeletal muscle homeostasis.

Here we demonstrate that local V1aR overexpression enhances regeneration in TNF-induced muscle atrophy. By modulating the inflammatory response, attenuating fibrogenesis, and upregulating the regeneration/differentiation markers, the stimulation of AVP-dependent pathways creates a favourable environment for sustained and efficient muscle regeneration and repair even in the presence of elevated levels of TNF. In addition, we demonstrate that the positive effect of V1aR overexpression in muscle homeostasis involves Akt-mediated inhibition of FoxO transcription factors.

The findings of this research are expected to lead to the identification of new pharmacological or gene therapy targets that may delay the progression of muscular wasting associated with numerous myopathies.

2. Materials and Methods

2.1. Animals. C57 transgenic desmin/nls-lacZ mice used in this study bear a transgene in which the 1-κB DNA 5′ regulatory sequence of the desmin gene is linked to a reporter gene coding for *Escherichia coli*-β-galactosidase [24]. The desmin/nls-lacZ transgene labels muscle cells in which the desmin synthesis program has commenced [25]. Mice were treated according to the guidelines of the Institutional Animal Care and Use Committee. They were housed in a temperature-controlled (22 ± 2°C) and humidity-controlled (60 ± 5%) room regulated to provide a 12 h light, 12 h dark cycle. Mice were allowed to feed and drink *ad libitum*. Animals were anesthetized with an intraperitoneal injection of Avertin A (a mix of tribromoethanol and 2-methyl-2-butanol diluted in physiological solution) before gene delivery by electroporation or muscle damage. Injury of mock-, V1aR-, TNF-, and V1aR+TNF-transfected *Tibialis anterior* (TA) muscles of 2-month-old mice was induced along the entire length of the muscle with a total of four cardiotoxin (CTX) injections (5 μL of 10 μM CTX per injection). In our previous study, regeneration was fully active at 7 days after injury [21].

Therefore, mice were sacrificed 7 days after injury and the regeneration process in the injured muscle was examined by assessing the number and size of regenerating fibers as previously described [21].

2.2. Plasmid Construction. The MLC-Myc-V1aR plasmid was derived from MLC-Myc and PCD3-V1aR (kindly provided by Prof. S.J. Lolait, University of Bristol, UK) expression vectors, as previously described by Toschi et al. [21]. To induce expression of the secreted form of murine TNF-α, we used the construct pBabe-mTNF-α (kindly provided by Dr. Gokhan Hotamisligil, Harvard University, Boston, MA) under control of the SV40 promoter. The SV40 promoter has been shown to be efficient for driving exogenous cDNA expression in skeletal muscle [26].

2.3. Gene Delivery by Electroporation. The TA in each mouse hind limb was injected with the indicated amount of cDNA: 20 μg of MLC-Myc-V1aR (V1aR), or pcDNA3 (mock) [21], or pBabe-mTNF-α (TNF)m or 10 μg of MLC-Myc-V1aR plus 10 μg of pBabe-mTNF-α (V1aR+TNF), in combination with 5 μg of pCMV-SNAP-GFP (kindly provided by Dr. Pozzan, University of Padua, Italy), as a marker of transfection efficiency. The electric pulses were delivered using 3 × 5 mm gene paddles electrodes (BTX, San Diego, CA) placed on either side of the muscle, as described by Donà et al. [27]. This protocol of gene delivery by electroporation guarantees stable DNA expression for more than four months. After electroporation, mice rapidly recovered and did not show any locomotor impairment or particular sign of pain or stress.

2.4. Histological and Histochemical Analysis. TA muscles from mock-, V1aR-, TNF or TNF+V1aR-transfected 7-8-week-old desmin/nls-lacZ mice were embedded in Jung tissue freezing medium (Leica, Wetzlar, Germany) and frozen in liquid nitrogen-cooled isopentane. Frozen sections (7 μm) were obtained using a Leica cryostat. Sections were observed under the green activation filter of the Axioskop 2 plus system (Zeiss). For histological analysis, sections were stained with hematoxylin and eosin (H&E) using standard methods [28].

Esterase staining was adapted from Davis [29] as previously reported [30]. Cryosections of each muscle were incubated for 5 min in a staining solution containing 3 mg alpha-naphthyl acetate, 0.375 mL acetone, 6.25 mL 0.2 M sodium phosphate, and 0.4 mL of a solution containing equal volumes of 2% pararosaniline (Sigma-Aldrich) and 2% sodium nitrite. Photomicrographs were obtained using an Axioscop2 plus system equipped with an AxiocamHRc (Zeiss, Oberkochen, Germany) at 1300 × 1030 pixel resolution and analyzed using 10x NA 0.30 air objective lens or 20x NA 0.50 air objective lens.

The trichrome stain (Masson) kit (Sigma-Aldrich Procedure number HT15) was used for distinguishing collagen from muscle tissue. Tissue sections were treated with Bouin's solution to intensify the final coloration. Nuclei were stained with Weigert's iron hematoxylin, and the cytoplasm and muscle were stained with Biebrich scarlet-acid fuchsin. After treatment with phosphotungstic and phosphomolybdic acid, collagen was demonstrated by staining with aniline blue.

2.5. Immunofluorescence Analysis. Frozen sections were fixed in 4% paraformaldehyde for 10 min on ice, washed with PBS, incubated in PBS containing 1% BSA (Sigma-Aldrich) and 1 : 100 goat serum for 30 min at room temperature, and then incubated overnight at 4°C with the selected primary antibody at the appropriate dilution. The following antibody was used: mouse monoclonal antiembryonic MHC (Developmental Hybridoma-Bank number BF-G6, University of Iowa, Iowa City, IA). Samples were then washed with PBS containing 1% BSA and incubated for 1 h at room temperature with the appropriate secondary antibody, Alexafluor 568-conjugated anti-mouse (Molecular Probes, Eugene, OR, USA) 1 : 500 in 1% BSA. Nuclei were visualized with Hoechst 33342 (Sigma-Aldrich). The sections were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and examined in a Zeiss Axioplan (Zeiss, Thornwood, NY) fluorescence microscopy using a 10x and 20x objective lens.

2.6. Morphometric Analysis. Photomicrographs of regenerating muscle fibers (identified by morphological criteria, i.e., centrally located nuclei in H&E stained sections) were taken at standard resolution (1.300 × 1030 pixel) and analyzed using ImageJ, Scion Image software. For morphometric evaluation of fiber size, 200–1000 cross-sectioned fibers per sample were analyzed. Quantitative data were obtained from three independent experiments in triplicate. The values are expressed as mean ± SD.

2.7. Gene Expression Analysis. TA muscles were dissected, and total RNA extraction was performed with tissue lyser (QIAGEN) in TriRagent (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol, and was reverse-transcribed using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT; Invitrogen, Carlsbad, CA). cDNA preamplification was performed with TaqMan PreAmp Master Mix Kit (Applied Biosystems) according to the manufacturer's instructions [31–33] to accurately detect the expression pattern of the following cytokines: IL4, IL10, IL6, IL1β, and the chemokine ligand CCL2 and scavenger receptor CD163. Quantitative PCR was performed on an ABI PRISM 7500 SDS (Applied Biosystems, USA), using premade 6-carboxyfluorescein (FAM)-labeled TaqMan assay for: TNF (Mm00443258_m1), V1aR (Mm00444092_m1), CCL2 (Mm00441242-m1), IL1-β (Mm01336189_m1), IL6 (Mm0120733_m1), CD163 (Mm004744096_m1), IL10 (Mm00439616_m1), IL-4 (Mm00445260_m1) Pax7 (Mm00834082_m1), desmin (Mm00802455_m1), myogenin (Mm00446194_m1), atrogin-1 (Mm01207878_m1), and HPRT (Mm00446968_m1) as internal controls. Real-time PCR was performed using RNA preparations from three different animals for each group.

2.8. Immunoblotting Analysis. TA muscles were dissected, minced, and homogenized with RIPA buffer (20 mMTris/HCl pH 7.5, 2 mM EDTA, 2 mM EGTA, 0.25 M Sucrose, 5 mM DTT, 0,1% Triton X-100, 10 mM NaF, 200 μM sodium orthovanadate) containing a protease inhibitor cocktail (Roche, Indianapolis, IN). Equal amounts of protein (20–30 μg), determined by Pierce BCA Protein Assay Reagents, were separated by SDS PAGE and transferred electrophoretically to a hybond-C extra nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). Nonspecific binding was blocked in Tris-buffered saline with Tween 20 (TBST) containing 5% nonfat milk for 1 h at room temperature, and the membrane was then incubated overnight at 4°C in TBST containing primary antibodies. The following antibodies were used: mouse monoclonal anti-MHC (Developmental Hybridoma-Bank), monoclonal anti-NFκB-p65 (C22B4), and monoclonal anti-phosphoNFκB-p65 (Ser563) (93H1) (Cell Signaling), rabbit polyclonal anti-IκBα (Santa Cruz Biotechnologies), polyclonal anti-phospho-Akt (Ser473), monoclonal anti-FoxO3a (75D8), and polyclonal anti-phospho-FoxO3a (Ser253) (Cell signaling). Monoclonal anti-tubulin-α (Sigma-Aldrich) or mouse monoclonal anti-GAPDH (Santa Cruz Biotechnologies) was used for normalization, as indicated. Blots were washed in TBST and then incubated with the appropriate secondary antibodies, goat anti-mouse, or anti-rabbit HRP-conjugated (Bio-Rad Laboratories, Hercules, CA) in TBST containing 1% nonfat milk. Blots were extensively washed and the antibody binding was detected by means of Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

2.9. Statistical Analysis. The Student's t-test was used throughout this paper for statistical analyses.

3. Results

3.1. Local V1aR Overexpression Counteracts the Negative Effects of TNF on Muscle Regeneration. Our previous data demonstrated that muscle specific overexpression of the AVP receptor V1a enhances skeletal muscle regeneration after CTX induced damage, by stimulating satellite cell activation and increasing the expression of differentiation markers [21]. We also demonstrated that TNF administration is sufficient

to induce inhibition of muscle regeneration by activating a nonapoptotic, caspase-dependent process, ultimately leading to muscle wasting [34]. Therefore, in this study we investigated whether local overexpression of V1aR protects the muscle from the effects of high TNF levels. To this end, we overexpressed the myosin-light-chain (MLC)-myc-V1a AVP receptor construct [21] alone or in combination with the TNF construct [21, 35] in TA muscles, by means of gene delivery by electroporation. Controls consisted of mock-transfected samples (i.e., muscles transfected with pcDNA3) and, when indicated, in nonelectroporated muscles (WT). In order to assess the efficacy of the transfections, we used real-time PCR analysis, to verify the V1aR and TNF expression levels in muscle extracts one week after electroporation (Figures 1(a) and 1(b)). V1aR expression was strongly upregulated in TA muscles electroporated with the V1aR construct, both in the presence and in the absence of TNF, demonstrating that TNF did not interfere with the electroporation efficiency or with the forced expression of V1aR (Figure 1(a)). As expected, TNF expression was high in samples electroporated with the TNF construct alone or in combination with V1aR (Figure 1(b)). The expression of V1aR and TNF did not differ significantly between the V1aR and V1aR+TNF samples (Figures 1(a) and 1(b)).

Morphological analysis showed that 1 week after electroporation, local overexpression of TNF (Figure 2(a), panel (B)) caused an accumulation of mononucleated infiltrating cells, compared with mock-transfected and V1aR overexpressing muscle alone (Figure 2(a), panels (A) and (C) resp.). In addition, a regeneration response occurred in the muscle rendered atrophic by TNF overexpression, as demonstrated by the presence of central nucleated fibers in TNF overexpressing muscle (Figure 2(b)). By contrast, cotransfection of V1aR and TNF did not significantly modify the number of regenerating fibers compared with TNF transfection alone. It did, however, affect the fiber size distribution by favoring the accumulation of larger fibers, as shown in Figure 2(c). It is noteworthy that the overexpression of V1aR induced a slight, yet significant increase in the fiber cross-sectional area compared with mock-transfected muscle (15% increase, $P < 0.001$) (data not shown). In conclusion, V1aR overexpression leads to more efficient regeneration than that observed in muscles overexpressing TNF alone.

3.2. Muscle V1aR Overexpression Modulates the Inflammatory Response and Attenuates the Fibrosis Induced by High Levels of TNF. TNF is a proinflammatory cytokine capable of activating macrophages, thereby inducing the production of other proinflammatory cytokines and perpetuating the inflammatory response. To verify whether the enhanced regenerative response in V1aR overexpressing muscle is associated with a modulation of inflammation, we examined the presence of macrophages by esterase staining. Figure 3(a) shows a high number of esterase-positive, macrophages in TNF overxpressing muscles, whereas the concomitant overexpression of V1aR significantly attenuated their number.

Efficient muscle repair is accompanied by and/or requires the migration and proliferation of fibroblasts needed to produce additional extracellular matrix (ECM) components acting as a scaffold for regenerating myofibers [3, 36, 37] and substituting the basement membrane and ECM components degraded during the inflammatory phases [38–40]. However, if inflammatory cell infiltration and fibroblast activation persist, an aberrant tissue repair response will produce a nonfunctional mass of fibrotic tissue [41]. In order to visualize the extent of fibrosis, we performed Masson's trichrome staining under the indicated experimental conditions. Figure 3(b) shows the normal presence of connective tissue in mock muscle and in muscle overexpressing V1aR, while muscles expressing TNF display increased amounts of ECM in skeletal muscle tissue. In the presence of high TNF and V1aR levels, the extent of fibrosis was significantly attenuated in comparison with muscle overexpressing TNF alone. Taken together, these data demonstrate that V1aR overexpression leads to a more efficient regeneration process, characterized by attenuated inflammatory response and reduced fibrosis.

3.3. V1aR Overexpression Modulates the Molecular Mechanisms Involved in the Inflammatory Response. It has been demonstrated that NF-κB is one of the central players of the inflammatory system [2, 42] and that TNF, along with other proinflammatory cytokines, stimulates the NF-κB signaling pathway, promoting muscle catabolism [5, 7]. Western blot and densitometric analysis (Figures 4(a), 4(b), and 4(c)) revealed that TNF overexpression promotes a strong upregulation of phospho-NF-κB expression and a concomitant downregulation of the NF-κB inhibitory factor, namely, IκBα. By contrast, phospho-NF-κB expression was significantly reduced in muscle overexpressing both TNF and V1aR, demonstrating that V1aR overexpression attenuates the effects of TNF on inflammation.

3.4. Inflammatory Cytokine Production Is Modulated in V1aR Overexpressing Muscle. To gain further insight into the mechanism by which V1aR modulates the resolution of inflammation and contributes to muscle repair, we performed real-time PCR, to analyse the expression patterns of specific cytokines and chemokines secreted by M1 macrophages, a proinflammatory cell population capable of perpetuating the inflammatory response [3]. Figure 5(a) demonstrates that the expression of CCL2, IL1β and IL6 was strongly upregulated in the presence of high levels of TNF compared with mock- and V1aR-transfected muscles. TNF overexpression was also accompanied by a slight increase in CD163 expression. By contrast, the overexpression of V1aR, in TNF expressed muscle, significantly reduced the expression of the proinflammatory cytokines. Interestingly, the reduction in M1 macrophages, promoted by V1aR overexpression, was associated with an upregulation of M2 macrophage markers such as CD163, IL-10, and IL-4 (Figure 5(b)). Notably, CD163 is a macrophage-specific receptor for complexes of hemoglobin and haptoglobin, and ligation of CD163 can contribute to the regulation of macrophage phenotype by increasing the expression of anti-inflammatory cytokines, especially IL-10 [43]. IL-4 is involved in muscle regeneration by promoting satellite cell fusion and differentiation [44–46], and we have

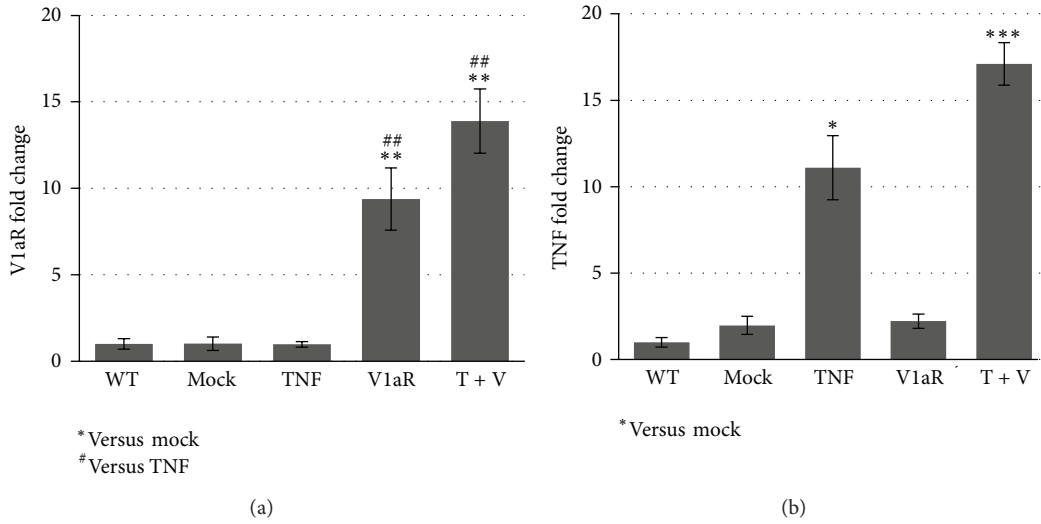


Figure 1: Expression of the transfected cDNA constructs is increased in electroporated muscles. Real-time PCR analysis of V1aR (a) and TNF (b) was performed on RNA extracts obtained from WT, mock-, V1aR-, TNF-, and V1aR+TNF-transfected TA muscles to verify the transfection efficiency after electroporation. The expression of V1aR and TNF is not significantly different when comparing V1aR versus V1aR+TNF and TNF versus V1aR+TNF samples, respectively (Figures 1(a) and 1(b)). Values represent the average three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ by Student's t-test.

previously demonstrated that stimulation of AVP signaling induces calcineurin-dependent expression of IL-4 in muscle cells. These results demonstrate that a high level of V1aR accelerates the resolution of TNF-dependent inflammation, modulating the muscle milieu by favoring the shift from M1 to M2 macrophage phenotype, thereby stimulating the secretion of anti-inflammatory cytokines and thus promoting regeneration.

3.5. Stimulation of AVP Signaling Modifies the Effects of TNF on Myogenesis and Promotes Muscle Maturation. Regeneration consists of a sequence of phenomena including the activation of satellite cells, and their differentiation and fusion into fibers expressing muscle specific products. Therefore, we first analyzed the expression of Pax7 and desmin, which are relevant markers of activated and proliferating satellite cells, 1 week after TNF and/or V1aR electroporation in muscles. Real-time PCR analysis demonstrated that TNF overexpression greatly increased the levels of Pax7 and desmin expression, when compared with WT and mock-transfected muscle, whereas the transfection of V1aR alone induced lower but significant changes in the expression of these proteins (Figures 6(a) and 6(b)). By contrast, the combination of V1aR and TNF overexpression promoted a significant increase in Pax7 and desmin expression. We then analyzed the expression of molecular markers characteristic of the terminal phases of muscle differentiation, such as myogenin and MHC. It is noteworthy that TNF overexpression downregulated the expression levels of MHC (but not of myogenin) compared with mock-transfected samples (Figure 6(c) and densitometry Figure 6(e)). By contrast, V1aR overexpression counteracted the negative effect of TNF on muscle differentiation, stimulating myogenin and MHC expression (Figures 6(c) and 6(d) and densitometry Figure 6(e)). Moreover, immunofluorescence analysis for embryonic-myosin heavy chain (embryonic-MHC), a marker of regenerating myofibers, revealed a significant increase in the number of embryonic-MHC positive fibers in muscles overexpressing both TNF and V1aR, compared with control and V1aR-alone transfected samples (Figure 6(f)).

Taken together, these results suggest that TNF stimulates satellite cell activation and muscle regeneration but, as expected, impinges on the maturation process. By contrast, overexpression of V1aR counteracts the negative effects of TNF, stimulating muscle growth and maturation.

3.6. V1aR Counteracts the Effect of TNF on Protein Degradation by Stimulating PI3K/Akt/FoxO Signaling. TNF is known to induce protein degradation as a result of activation of the ubiquitin-dependent proteasome pathway [7]. On the other hand, the PI3K/Akt signaling is one of the most critical pathways involved in the regulation of skeletal muscle mass [8, 12]. Akt phosphorylates FoxO transcription factors in multiple sites, leading to the exclusion of phosphorylated FoxO proteins from the nucleus and inhibition of atrogin-1 expression [47]. We therefore analyzed the effect of TNF on Akt and FoxO3a expression in the presence and absence of V1aR overexpression. Here we demonstrate that, in muscle overexpressing TNF, the phosphorylated form of Akt is downregulated compared with mock-transfected muscle, while the expression of native FoxO (FoxO3a) is high. The double band of native FoxO3a is ascribable to the presence of posttranslational modifications of FoxO3a, such as acetylation or ubiquitination. By contrast, phospho-Akt is upregulated in V1aR overexpressing muscles, either alone or in combination with a high TNF level (Figure 7(a)). Indeed, phospho-FoxO3a is downregulated only in samples

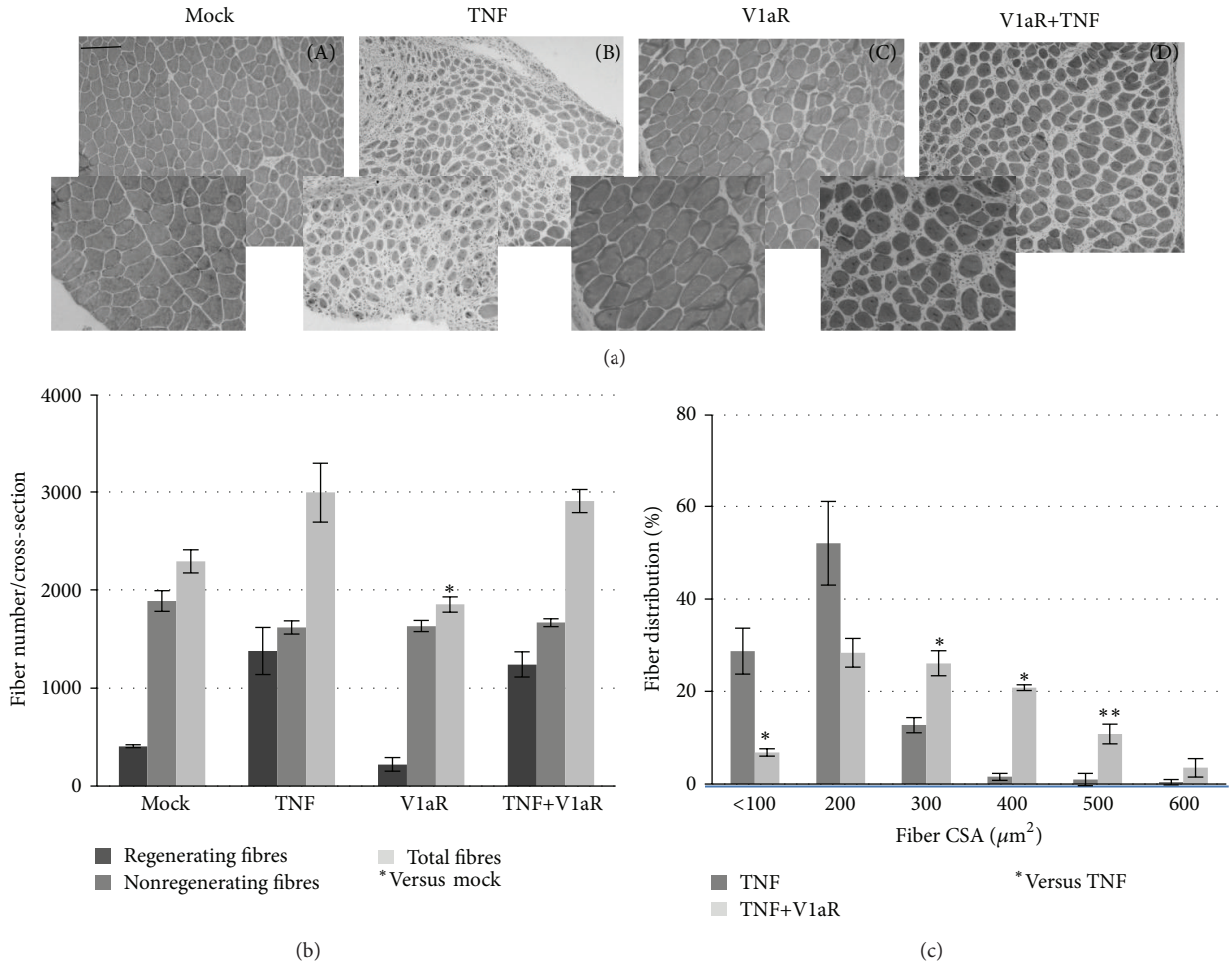


Figure 2: Local V1aR overexpression in atrophic muscle enhances the regeneration process. (a) Cross-sections of mock (A), TNF- (B), V1aR- (C), and TNF+V1aR- (D) transfected TA were stained with H&E one week after electroporation. Scale bar = 50 μm. Original magnification 10x and insert magnification 20x. (b) Diagram showing the number of regenerating, nonregenerating, and total fibers per cross-sectional area. $^{*}P < 0.05$. (c) Morphometric analysis shows that in muscles overexpressing V1aR and TNF together the mean fiber cross sectional area (CSA) is bigger compared with samples overexpressing TNF alone. $^{*}P < 0.05$; $^{**}P < 0.01$ by Student's t-test.

overexpressing TNF alone, and the expression of atrogin-1 in this condition is strongly upregulated (Figures 7(a) and 7(e)). These results demonstrate that V1aR overexpression stimulates the PI3K/Akt pathways leading to phosphorylation of FoxO transcription factors, which in turn results in downregulation of atrogin-1 expression.

4. Discussion

In this study, we show that the negative effects of TNF on muscle regeneration and inflammation are strongly counteracted by stimulation of AVP signaling. AVP, a neurohypophyseal nonapeptide, is a potent myogenesis-promoting factor both *in vitro* and *in vivo*. By interacting with V1aR, AVP increases cytosolic Ca^{2+} concentrations, upregulates Myf-5 and myogenin expression, and activates $Ca2^{+}$/calmodulin-dependent protein kinase (CaMK) and CnA signaling pathways [16, 17, 48–51]. Moreover, local V1aR overexpression results in acceleration of the regeneration process, as demonstrated by rapid resolution of inflammation, earlier activation and fusion of satellite cells, and formation of regenerating fibers compared with the mock-transfected muscles [21].

To better clarify the molecular pathways involved in the positive effects of V1aR overexpression in muscle homeostasis, we induced morphological alterations in TA muscles by local overexpression of TNF and analyzed the effects of high levels of V1aR on TNF-induced muscle atrophy.

TNF is a proinflammatory cytokine known to induce murine myoblast apoptosis [52] and block human muscle satellite cell differentiation [53]. Chronic exposure to low levels of circulating TNF inhibits muscle regeneration and induces cachexia [35, 54].

Morphological analysis clearly demonstrated that the accumulation of infiltrating cells in muscle overexpressing TNF alone dramatically decreases when TNF and V1aR are overexpressed together. Moreover, while V1aR does not significantly modify the number of regenerating fibers compared with samples overexpressing TNF alone,

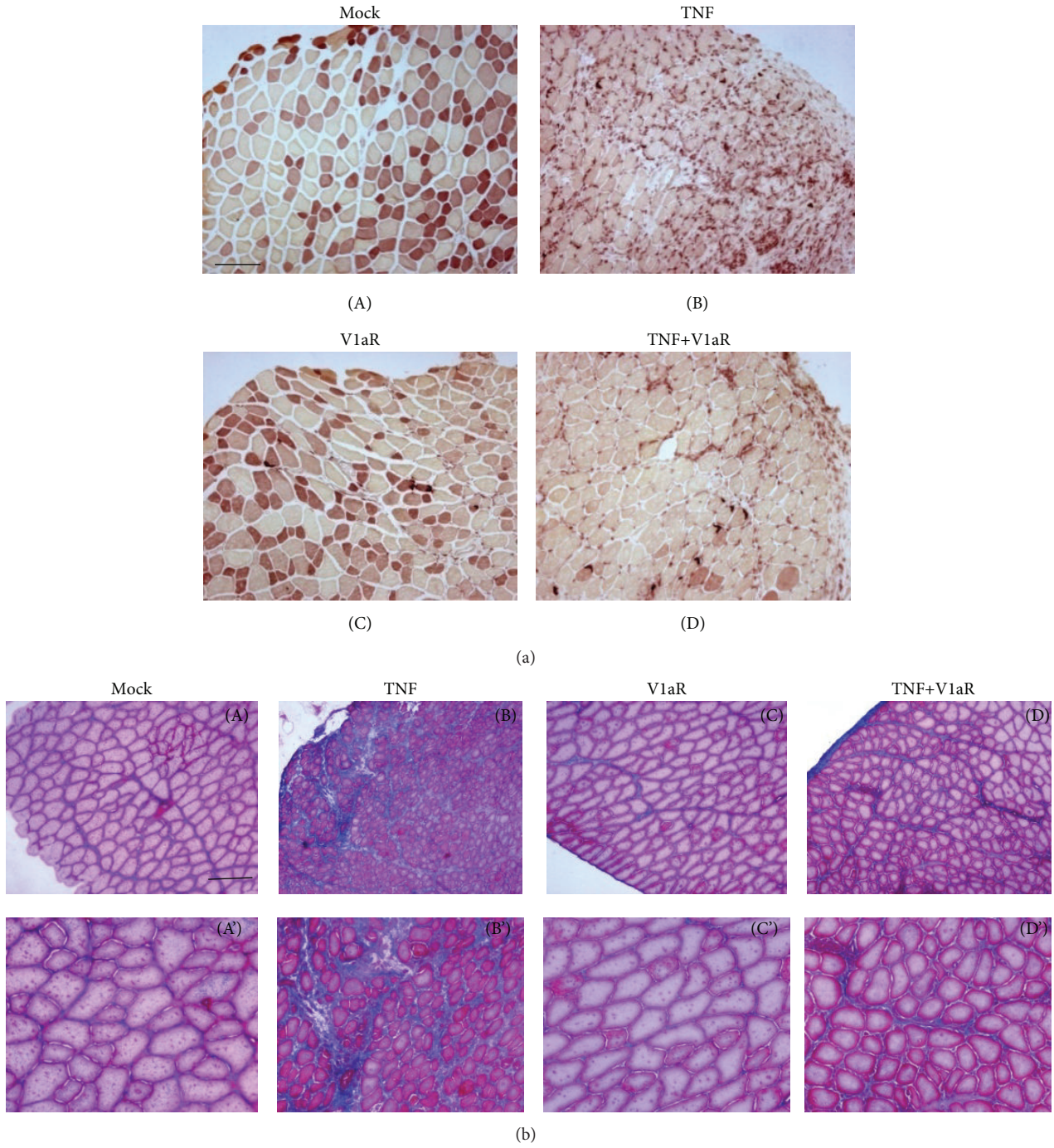


Figure 3: V1aR overexpression attenuates inflammation and fibrosis induced by high levels of TNF. (a) Nonspecific esterase staining of TA cross-sections, performed one week after electroporation. The figure highlights the massive presence of macrophages in muscle overexpressing TNF (B) and a reduced esterase activity in muscle overexpressing both TNF and V1aR (D). (Scale bar = 50 μm, magnification 10x.) (b) Masson's trichrome stain of TA cross-sections, one week after electroporation, demonstrates less extensive fibrosis in muscle overexpressing TNF+V1aR (D and D'), compared with muscle overexpressing TNF alone (B and B'). (Scale bar = 50 μm, magnification 10x, (A)–(D); 20x, (A')–(D').

it does affect the fiber size distribution suggesting that it may play a role in acceleration of the regeneration process. Furthermore, a comparison between mock and V1aR-transfected muscles indicated that V1aR overexpression exerts a hypertrophic effect on the fibers, regardless of the regeneration process, which may be attributable to either increased fusion of satellite cells or increased synthesis and accumulation of contractile proteins. Inflammation is a critical component of muscle physiology that represents an important phase in regeneration and is often associated with severe and progressive fibrosis [3, 36, 37, 55]. Invasion by neutrophils and macrophages characterizes

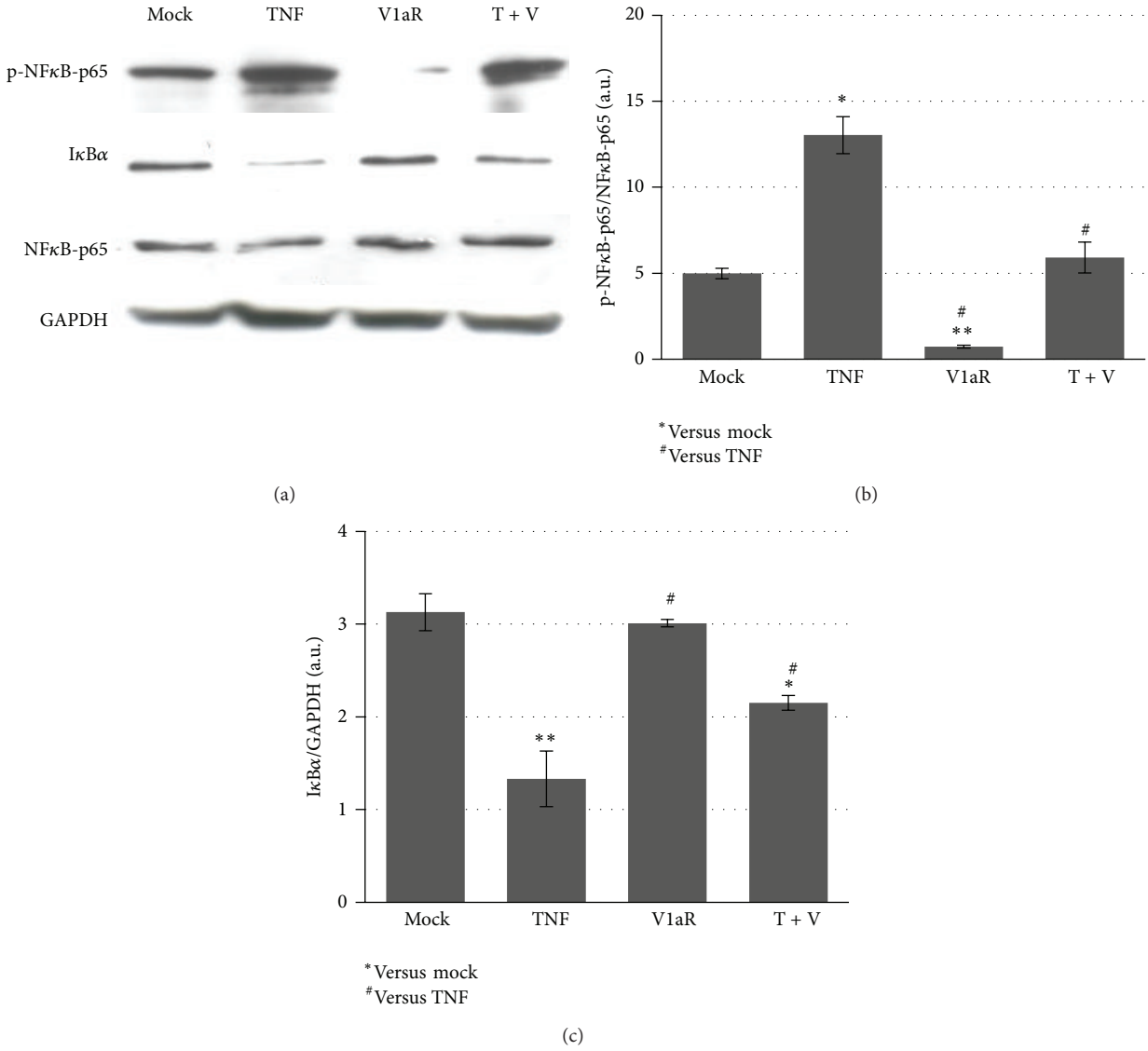


Figure 4: Downregulation of NF-κB pathway in muscle over-expressing V1aR. (a) Western blot analysis revealed that TNF overexpression promotes downregulation of IκBα with concomitant upregulation of the phosphorylated form of NFκB (p-NF-κB-p65) expression. By contrast, p-NF-κB-p65 expression was significantly reduced in muscle overexpressing TNF and V1aR together compared with TNF alone. The expression of native NF-κB-p65 does not reveal significant differences between the various samples. (b, c) Densitometric analysis of two independent experiments for phospho-NF-κB-p65 versus native NF-κB-p65 (b) and IκBα versus GAPDH (c) expression. $^{*\#}P < 0.05$; $^{**}P < 0.01$ by Student's t-test.

the initial phases of inflammation. This is followed by downregulation of the inflammatory response, thus preventing further damage and favouring regeneration [56]. The shift from the M1-macrophage-dependent necrotic environment to the M2-macrophage phase characterized by stem cell recruitment and differentiation is important insofar as it promotes regeneration [57, 58]. This could also explain the slight increase in CD163 (involved in anti-inflammatory responses) expression occurring in TNF overexpressing muscles [2, 3]. Our data are in line with recent findings demonstrating that the V1aR agonist dramatically reduces the mRNA level of proinflammatory cytokines in astrocytes [59].

The enhanced regenerative capacity of V1aR overexpressing muscles correlated with dampening of inflammation and connective tissue accumulation; consequently, fibrosis and prolonged infiltration by monocytes/macrophages typical of TNF overexpressing muscle were more rapidly resolved in the presence of high levels of V1aR. This finding is supported by the V1aR-dependent modulation of NF-κB expression, one of the most important molecular players in the activation and maintenance of inflammation in response to TNF stimuli [9, 60].

Local overexpression of V1aR selectively downregulated the proinflammatory cytokines, CCL2, IL1β, IL-6, whose

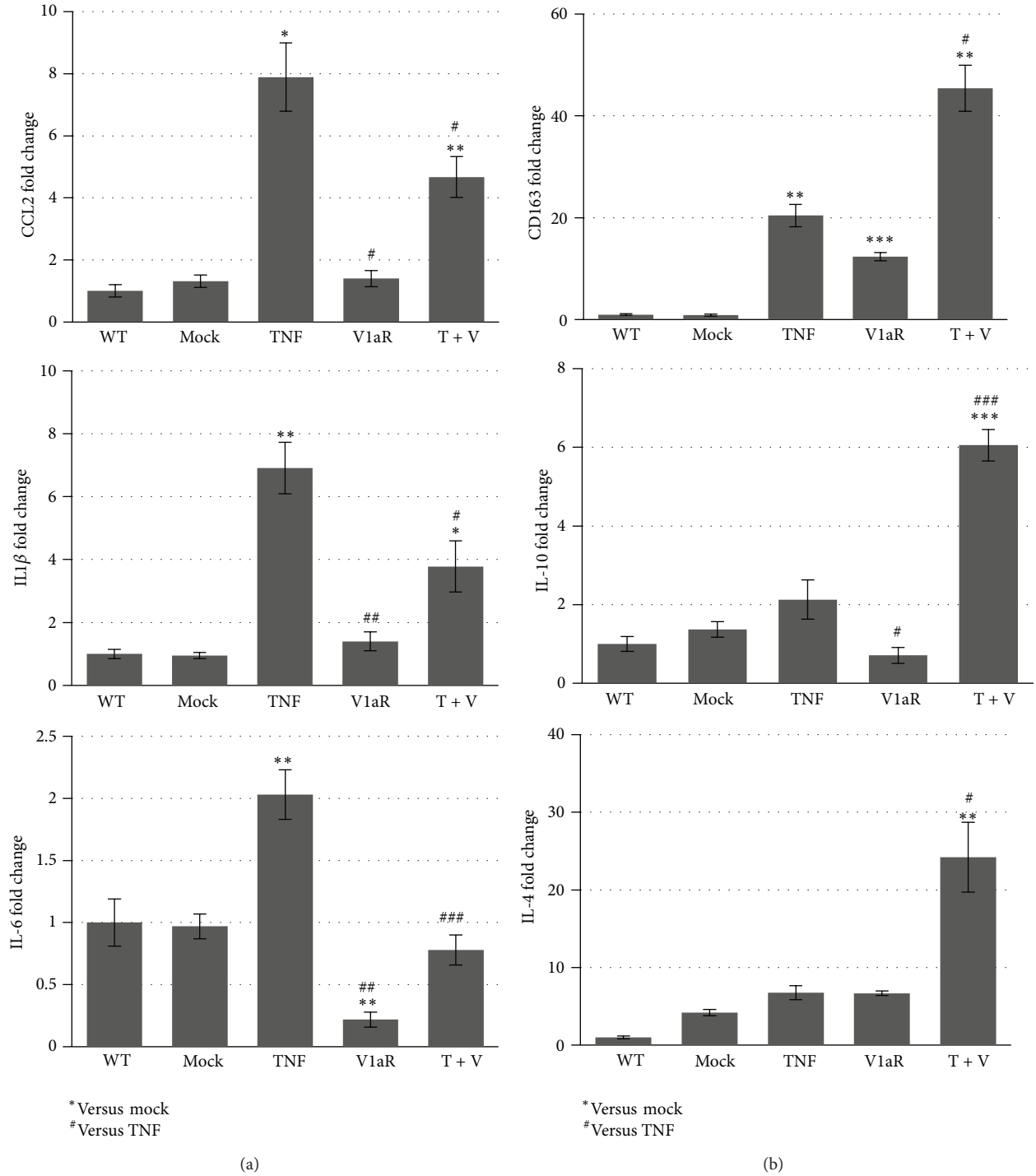


Figure 5: V1aR overexpression directs inflammation towards the repair phase. (a) Real-time PCR analysis for CCL2, IL-1β, and IL-6. These chemokines and cytokines typical of the first proinflammatory phase were highly transcribed in muscle overexpressing TNF alone, while the coexpression of V1aR significantly reduced their expression. By contrast, the expression levels of the anti-inflammatory cytokines CD163, IL-10, and IL-4 (b) were strongly induced in muscle overexpressing V1aR and TNF together. (b) $^{*,\#}P < 0.05$; $^{**,\#\#}P < 0.01$; $^{***,\#\#\#}P < 0.001$ by Student's t-test.

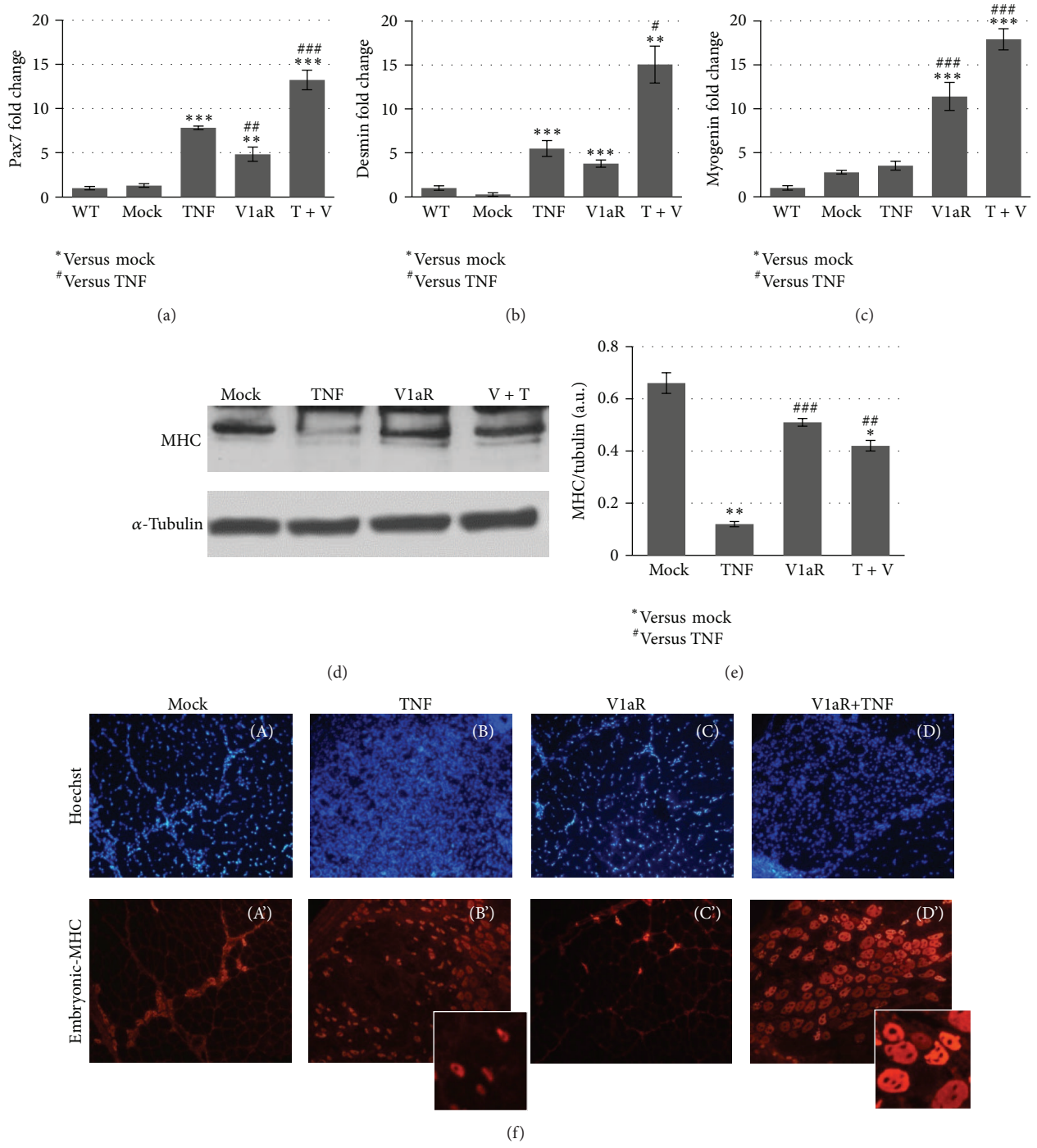


Figure 6: V1aR overexpression induces muscle regeneration even in the presence of TNF. Real-time PCR analysis shows that forced expression of TNF significantly increases the expression of Pax7 (a) and desmin (b) compared with mock-transfected samples. The expression level of these markers is even higher in muscle overexpressing TNF and V1aR together, while only a slight, yet significant, increase is observed in V1aR-transfected muscle. Interestingly, myogenin expression (c) does not significantly change in the presence of TNF, while it is strongly upregulated in V1aR overexpressing muscles, both in the presence and in the absence of TNF. (d) Western blot analysis shows a downregulation of MHC expression by TNF, while its expression increases in V1aR and V1aR+TNF overexpressing muscles. (e) Densitometric analysis of three independent experiments for MHC versus tubulin expression. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ by Student's t-test. (f) Immunofluorescence analysis of embryonic MHC in TA cross-sections. Muscles overexpressing both TNF and V1aR display an increase in number and size of regenerating muscle fibers compared with TNF overexpressing muscle.

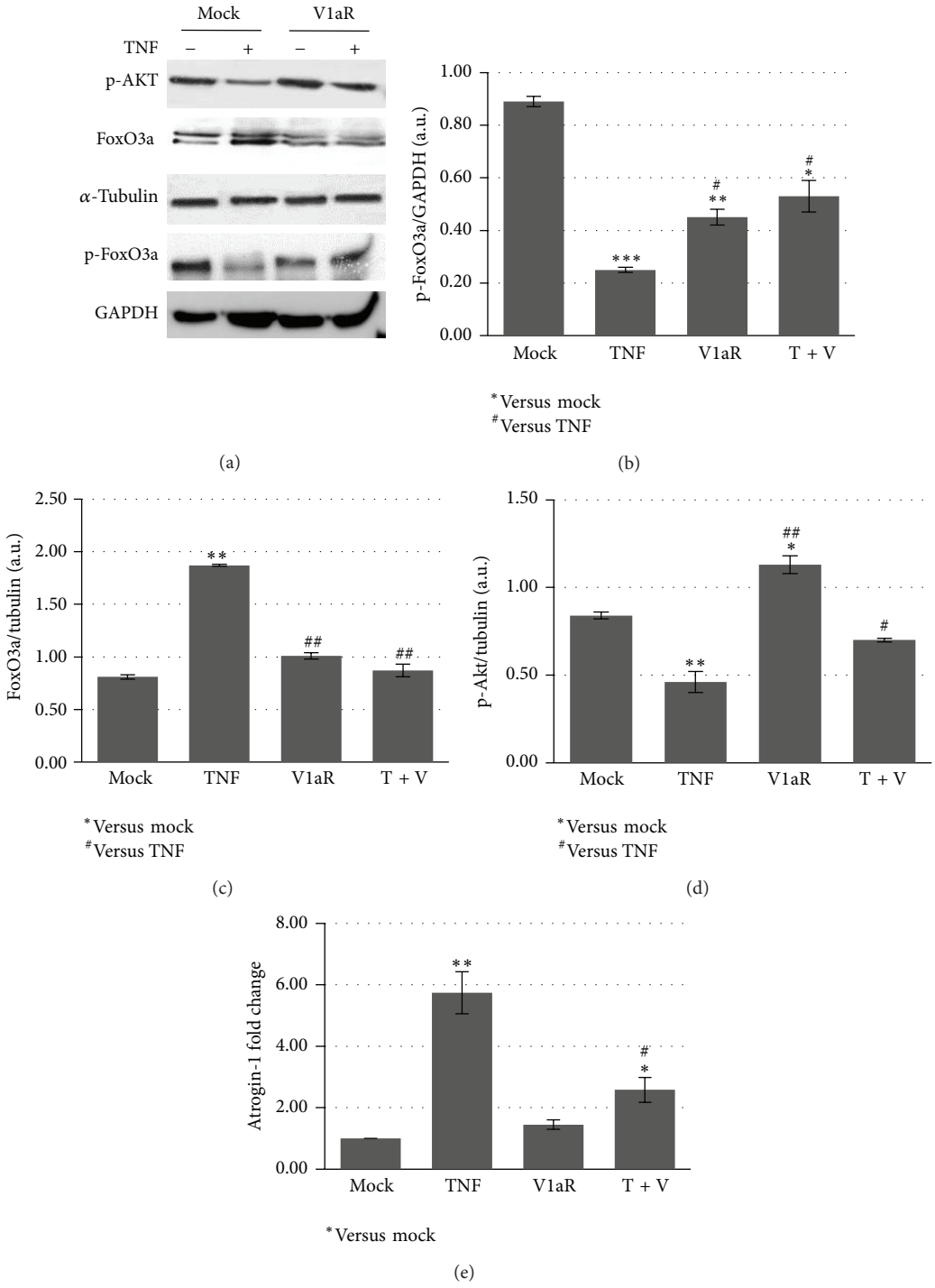


FIGURE 7: V1aR overexpression counteracts TNF-dependent protein degradation by stimulating the Akt pathway. (a) Western blots of phosphorylated Akt and native and phosphorylated FoxO3a expression demonstrate that in muscle overexpressing TNF, phospho-Akt and phospho-FoxO3a are downregulated, while the native Foxo3a is increased. In V1aR overexpressing muscles, the expression levels of phospho-FoxO3a and phospho-Akt is increased compared with TNF alone, while the native Foxo3a is reduced. (b–d) Densitometric analysis of three independent experiments of phospho-Akt, phospho-FoxO3a, and native FoxO3a expression levels. (e) Real-time PCR analysis revealed that the strong upregulation of atrogin-1 expression observed in the sample overexpressing TNF alone is downregulated in V1aR+TNF-transfected muscles. $^{*}P < 0.05$; $^{**}P < 0.01$ by Student's t-test.

expression is associated with sustained mononuclear cell influx and the switch from acute to chronic inflammatory process [3, 5]. Interestingly, V1aR upregulated the expression of anti-inflammatory cytokines, including CD163, IL-10, and IL-4, which play a major role in promoting growth and regeneration [61]. In particular, several works have identified IL-4 as a key cytokine in myogenic fusion processes. Being directly correlated with CnA activity, IL-4 is synthesized and secreted after the translocation of nuclear factor of activated T-cells into the nucleus and stimulates fusion of myoblasts to preformed myotubes [44–46]. Moreover, we previously demonstrated that IL-4 represents a key cytokine in the mediation of the effects of AVP on skeletal muscle homeostasis [21]. In the present study we found that TNF stimulates satellite cell activation and muscle regeneration, as demonstrated by the upregulation of Pax7 and desmin expression, but, as expected, impinges on the maturation process as shown by the low levels of the late differentiation marker MHC. By contrast, the overexpression of V1aR counteracted the negative effects of TNF, stimulating muscle growth and maturation as demonstrated by the increased expression levels of myogenin and MHC in TNF+V1aR overexpressing muscles.

One of the most important mechanisms controlling cellular and protein turnover is mediated by Akt-FoxO. A reduction in the activity of the Akt pathway, as observed in different models of muscular atrophy, results in decreased levels of phosphorylated FoxO and consequent upregulation of atrophy related genes [14, 62], which are responsible for increased protein degradation through the ubiquitin-proteasome system [7, 63, 64]. Here we demonstrate that TNF overexpression upregulates dephosphorylated FoxO expression, thus promoting the transcriptional activation of atrogin-1. By contrast, V1aR overexpression stimulated the PI3K/Akt pathways, leading to phosphorylation of FoxO transcription factors and resulting in the inhibition of atrogin-1 expression.

Since neurohypophyseal hormones are not canonical regulators of skeletal muscle structure and function, the physiological relevance of our findings merits discussion. A large body of evidence indicates that AVP and/or OT play a significant role in promoting differentiation and hypertrophy of myogenic cells in culture. Recently, Breton et al. provided evidence that functional oxytocin receptors are present in human primary myoblasts [19]. The finding that levels of immunoreactive AVP, which are high in embryonic skeletal muscle, decline during gestation and reach very low levels at birth points to a role of AVP in muscle development [65]. These data, combined with our results demonstrating a modulation of V1aR endogenous expression during the regeneration process, suggest that AVP signaling plays a significant role in skeletal muscle homeostasis [21]. Interestingly, several authors have shown that exercise, a physiological hypertrophic cue, significantly increases circulating AVP, both in humans and in other mammals, thus posing the theoretical basis for the physiological regulation of muscle hypertrophy by neurohypophyseal hormones [49, 66–69]. De Jager et al. recently reported that treatment of cattle with anabolic steroids unexpectedly led to a high expression of mRNA encoding oxytocin in muscle, accompanied by a high level of circulating oxytocin in the plasma [70], suggesting that OT is involved in mediating the anabolic effects of the treatment.

5. Conclusions

Our findings show that the stimulation of AVP signaling in muscle enhances the regeneration process by attenuating inflammation and fibrosis and by modulating protein degradation. Stimulation of AVP signaling might represent an interesting novel strategy to counteract muscle decline in aging or in muscular pathologies.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors are indebted to Dr. Biliana Lozanoska-Ochser for critically revising the paper. This paper is supported in part by PRIN 2009 WBFZYM and by 2012 N8YJC3 and by Sapienza University Project C26A12ENW, to S.A.

References

[1] S. Gordon and P. R. Taylor, "Monocyte and macrophage heterogeneity," *Nature Reviews Immunology*, vol. 5, no. 12, pp. 953–964, 2005.

[2] J. G. Tidball, "Inflammatory processes in muscle injury and repair," *American Journal of Physiology: Regulatory Integrative and Comparative Physiology*, vol. 288, no. 2, pp. R345–R353, 2005.

[3] Y. Kharraz, J. Guerra, C. J. Mann, A. L. Serrano, and P. Muñoz-Cánoves, "Macrophage plasticity and the role of inflammation in skeletal muscle repair," *Mediators of Inflammation*, vol. 2013, Article ID 491497, 9 pages, 2013.

[4] M. Saclier, S. Cuvellier, M. Magnan, R. Mounier, and B. Chazaud, "Monocyte/macrophage interactions with myogenic precursor cells during skeletal muscle regeneration," *The FEBS Journal*, vol. 280, no. 17, pp. 4118–4130, 2013.

[5] M. R. Douglas, K. E. Morrison, M. Salmon, and C. D. Buckley, "Why does inflammation persist: a dominant role for the stromal microenvironment?" *Expert Reviews in Molecular Medicine*, vol. 4, no. 25, pp. 1–18, 2002.

[6] S. Carosio, M. G. Berardinelli, M. Aucello, and A. Musarò, "Impact of ageing on muscle cell regeneration," *Ageing Research Reviews*, vol. 10, no. 1, pp. 35–42, 2011.

[7] Y.-P. Li and M. B. Reid, "NF-κB mediates the protein loss induced by TNF-α in differentiated skeletal muscle myotubes," *American Journal of Physiology: Regulatory Integrative and Comparative Physiology*, vol. 279, no. 4, pp. R1165–R1170, 2000.

[8] S. Schiaffino, K. A. Dyar, S. Ciciliot, B. Blaauw, and M. Sandri, "Mechanisms regulating skeletal muscle growth and atrophy," *The FEBS Journal*, vol. 280, no. 17, pp. 4294–4314, 2013.

[9] A. Kumar, Y. Takada, A. M. Boriek, and B. B. Aggarwal, "Nuclear factor-κB: its role in health and disease," *Journal of Molecular Medicine*, vol. 82, no. 7, pp. 434–448, 2004.

[10] M. S. Hayden and S. Ghosh, "Shared principles in NF-κB signaling," *Cell*, vol. 132, no. 3, pp. 344–362, 2008.

[11] F. Mourkioti, P. Kratsios, T. Luedde et al., "Targeted ablation of IKK2 improves skeletal muscle strength, maintains mass, and promotes regeneration," *The Journal of Clinical Investigation*, vol. 116, no. 11, pp. 2945–2954, 2006.

[12] P. Bonaldo and M. Sandri, "Cellular and molecular mechanisms of muscle atrophy," *Disease Models & Mechanisms*, vol. 6, no. 1, pp. 25–39, 2013.

[13] C.-M. Liu, Z. Yang, C.-W. Liu et al., "Effect of RNA oligonucleotide targeting Foxo-1 on muscle growth in normal and cancer cachexia mice," *Cancer Gene Therapy*, vol. 14, no. 12, pp. 945–952, 2007.

[14] M. Sandri, C. Sandri, A. Gilbert et al., "Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy," *Cell*, vol. 117, no. 3, pp. 399–412, 2004.

[15] S. Minotti, B. M. Scicchitano, C. Nervi et al., "Vasopressin and insulin-like growth factors synergistically induce myogenesis in serum-free medium," *Cell Growth & Differentiation*, vol. 9, no. 2, pp. 155–163, 1998.

[16] C. Nervi, L. Benedetti, A. Minasi, M. Molinaro, and S. Adamo, "Arginine-vasopressin induces differentiation of skeletal myogenic cells and up-regulation of myogenin and Myf-5," *Cell Growth & Differentiation*, vol. 6, no. 1, pp. 81–89, 1995.

[17] A. Teti, F. Naro, M. Molinaro, and S. Adamo, "Transduction of arginine vasopressin signal in skeletal myogenic cells," *American Journal of Physiology: Cell Physiology*, vol. 265, no. 1, part 1, pp. C113–C121, 1993.

[18] M. Alvisi, V. de Arcangelis, L. Ciccone et al., "V1a vasopressin receptor expression is modulated during myogenic differentiation," *Differentiation*, vol. 76, no. 4, pp. 371–380, 2008.

[19] C. Breton, C. Haenggeli, C. Barberis et al., "Presence of functional oxytocin receptors in cultured human myoblasts," *The Journal of Clinical Endocrinology & Metabolism*, vol. 87, no. 3, pp. 1415–1418, 2002.

[20] M. Thibonnier, M. K. Graves, M. S. Wagner, C. Auzan, E. Clauser, and H. F. Willard, "Structure, sequence, expression, and chromosomal localization of the human V_{1a} vasopressin receptor gene," *Genomics*, vol. 31, no. 3, pp. 327–334, 1996.

[21] A. Toschi, A. Severi, D. Coletti et al., "Skeletal muscle regeneration in mice is stimulated by local overexpression of V1a-vasopressin receptor," *Molecular Endocrinology*, vol. 25, no. 9, pp. 1661–1673, 2011.

[22] J.-L. G. de Aguilar, J. W. Gordon, F. René, B. Lutz-Bucher, P. Kienlen-Campard, and J.-P. Loeffler, "A mouse model of familial amyotrophic lateral sclerosis expressing a mutant superoxide dismutase 1 shows evidence of disordered transport in the vasopressin hypothalamo-neurohypophysial axis," *European Journal of Neuroscience*, vol. 11, no. 12, pp. 4179–4187, 1999.

[23] D. Michelson, L. Stone, E. Galliven et al., "Multiple sclerosis is associated with alterations in hypothalamic-pituitary-adrenal axis function," *The Journal of Clinical Endocrinology & Metabolism*, vol. 79, no. 3, pp. 848–853, 1994.

[24] L. Lescaudron, Z. Li, D. Paulin, and J. Fontaine-Perus, "Desmin-lacZ transgene, a marker of regenerating skeletal muscle," *Neuromuscular Disorders*, vol. 3, no. 5-6, pp. 419–422, 1993.

[25] L. Lescaudron, S. E. Creuzet, Z. Li, D. Paulin, and J. Fontaine-Pérus, "Desmin-lacZ transgene expression and regeneration within skeletal muscle transplants," *Journal of Muscle Research and Cell Motility*, vol. 18, no. 6, pp. 631–641, 1997.

[26] V. A. Lupa-Kimball and K. A. Esser, "Use of DNA injection for identification of slow nerve-dependent regions of the MLC2s gene," *American Journal of Physiology: Cell Physiology*, vol. 274, no. 1, part 1, pp. C229–C235, 1998.

[27] M. Donà, M. Sandri, K. Rossini, I. Dell'Aica, M. Podhorska-Okolow, and U. Carraro, "Functional in vivo gene transfer into the myofibers of adult skeletal muscle," *Biochemical and Biophysical Research Communications*, vol. 312, no. 4, pp. 1132–1138, 2003.

[28] A. H. Fischer, K. A. Jacobson, J. Rose, and R. Zeller, "Hematoxylin and eosin staining of tissueand cell sections," *Cold Spring Harbor Protocols*, vol. 3, no. 5, 2008.

[29] B. J. Davis, "Histochemical demonstration of erythrocyte esterases," *Experimental Biology and Medicine*, vol. 101, no. 1, pp. 90–93, 1959.

[30] E. Berardi, P. Aulino, I. Murfuni et al., "Skeletal muscle is enriched in hematopoietic stem cells and not inflammatory cells in cachectic mice," *Neurological Research*, vol. 30, no. 2, pp. 160–169, 2008.

[31] I. Desitter, B. S. Guerrouahen, N. Benali-Furet et al., "A new device for rapid isolation by size and characterization of rare circulating tumor cells," *Anticancer Research*, vol. 31, no. 2, pp. 427–441, 2011.

[32] A. London, E. Itskovich, I. Benhar et al., "Neuroprotection and progenitor cell renewal in the injured adult murine retina requires healing monocyte-derived macrophages," *The Journal of Experimental Medicine*, vol. 208, no. 1, pp. 23–39, 2011.

[33] A. Turchinovich, L. Weiz, A. Langheinz, and B. Burwinkel, "Characterization of extracellular circulating microRNA," *Nucleic Acids Research*, vol. 39, no. 16, pp. 7223–7233, 2011.

[34] V. Moresi, A. Pristerà, B. M. Scicchitano et al., "Tumor necrosis factor-α inhibition of skeletal muscle regeneration is mediated by a caspase-dependent stem cell response," *Stem Cells*, vol. 26, no. 4, pp. 997–1008, 2008.

[35] D. Coletti, V. Moresi, S. Adamo, M. Molinaro, and D. Sassoon, "Tumor necrosis factor-α gene transfer induces cachexia and inhibits muscle regeneration," *Genesis*, vol. 43, no. 3, pp. 120–128, 2005.

[36] C. M. Murphy, A. Matsiko, M. G. Haugh, J. P. Gleeson, and F. J. O'Brien, "Mesenchymal stem cell fate is regulated by the composition and mechanical properties of collagen-glycosaminoglycan scaffolds," *Journal of the Mechanical Behavior of Biomedical Materials*, vol. 11, pp. 53–62, 2012.

[37] A. L. Serrano, C. J. Mann, B. Vidal, E. Ardite, E. Perdiguero, and P. Muñoz-Cánoves, "Cellular and molecular mechanisms regulating fibrosis in skeletal muscle repair and disease," *Current Topics in Developmental Biology*, vol. 96, pp. 167–201, 2011.

[38] A. K. Gulati, A. H. Reddi, and A. A. Zalewski, "Changes in the basement membrane zone components during skeletal muscle fiber degeneration and regeneration," *The Journal of Cell Biology*, vol. 97, no. 4, pp. 957–962, 1983.

[39] V. Moresi, G. Garcia-Alvarez, A. Pristerà et al., "Modulation of caspase activity regulates skeletal muscle regeneration and function in response to vasopressin and tumor necrosis factor," *PLoS ONE*, vol. 4, no. 5, Article ID e5570, 2009.

[40] C. M. Overall and C. P. Blobel, "In search of partners: linking extracellular proteases to substrates," *Nature Reviews Molecular Cell Biology*, vol. 8, no. 3, pp. 245–257, 2007.

[41] C. J. Mann, E. Perdiguero, Y. Kharraz et al., "Aberrant repair and fibrosis development in skeletal muscle," *Skeletal Muscle*, vol. 1, article 21, 2011.

[42] C. Smith, M. J. Kruger, R. M. Smith, and K. H. Myburgh, "The inflammatory response to skeletal muscle injury: illuminating complexities," *Sports Medicine*, vol. 38, no. 11, pp. 947–969, 2008.

[43] K. Yunoki, T. Inoue, K. Sugioka et al., "Association between hemoglobin scavenger receptor and heme oxygenase-1-related anti-inflammatory mediators in human coronary stable and unstable plaques," *Human Pathology*, vol. 44, no. 10, pp. 2256–2265, 2013.

[44] S. Chargé and M. A. Rudnicki, "Fusion with the fused: a new role for interleukin-4 in the building of muscle," *Cell*, vol. 113, no. 4, pp. 422–423, 2003.

[45] V. Horsley, K. M. Jansen, S. T. Mills, and G. K. Pavlath, "IL-4 acts as a myoblast recruitment factor during mammalian muscle growth," *Cell*, vol. 113, no. 4, pp. 483–494, 2003.

[46] G. K. Pavlath and V. Horsley, "Cell fusion in skeletal muscle: central role of NFATC2 in regulating muscle cell size," *Cell Cycle*, vol. 2, no. 5, pp. 419–422, 2003.

[47] E. Andres-Mateos, H. Brinkmeier, T. N. Burks et al., "Activation of serum/glucocorticoid-induced kinase 1 (SGK1) is important to maintain skeletal muscle homeostasis and prevent atrophy," *EMBO Molecular Medicine*, vol. 5, no. 1, pp. 80–91, 2013.

[48] B. M. Scicchitano, L. Spath, A. Musarò, M. Molinaro, S. Adamo, and C. Nervi, "AVP induces myogenesis through the transcriptional activation of the myocyte enhancer factor 2," *Molecular Endocrinology*, vol. 16, no. 6, pp. 1407–1416, 2002.

[49] B. M. Scicchitano, L. Spath, A. Musarò et al., "Vasopressin-dependent myogenic cell differentiation is mediated by both Ca^{2+}/calmodulin-dependent kinase and calcineurin pathways," *Molecular Biology of the Cell*, vol. 16, no. 8, pp. 3632–3641, 2005.

[50] V. de Arcangelis, D. Coletti, M. Canato et al., "Hypertrophy and transcriptional regulation induced in myogenic cell line L6-C5 by an increase of extracellular calcium," *Journal of Cellular Physiology*, vol. 202, no. 3, pp. 787–795, 2005.

[51] F. Naro, V. de Arcangelis, D. Coletti et al., "Increase in cytosolic Ca^{2+} induced by elevation of extracellular Ca^{2+} in skeletal myogenic cells," *American Journal of Physiology: Cell Physiology*, vol. 284, no. 4, pp. C969–C976, 2003.

[52] C. E. H. Stewart, P. V. Newcomb, and J. M. P. Holly, "Multifaceted roles of TNF-α in myoblast destruction: a multitude of signal transduction pathways," *Journal of Cellular Physiology*, vol. 198, no. 2, pp. 237–247, 2004.

[53] E. J. Foulstone, C. Huser, A. L. Crown, J. M. P. Holly, and C. E. H. Stewart, "Differential signalling mechanisms predisposing primary human skeletal muscle cells to altered proliferation and differentiation: roles of IGF-I and TNFα," *Experimental Cell Research*, vol. 294, no. 1, pp. 223–235, 2004.

[54] D. Coletti, L. Belli, and S. Adamo, "Cachexia: novel perspectives for an old syndrome," *Basic Applied Myology*, vol. 16, no. 5-6, pp. 131–139, 2006.

[55] C. M. Hogaboam, M. L. Steinhauser, S. W. Chensue, and S. L. Kunkel, "Novel roles for chemokines and fibroblasts in interstitial fibrosis," *Kidney International*, vol. 54, no. 6, pp. 2152–2159, 1998.

[56] L. Pelosi, C. Giacinti, C. Nardis et al., "Local expression of IGF-1 accelerates muscle regeneration by rapidly modulating inflammatory cytokines and chemokines," *The FASEB Journal*, vol. 21, no. 7, pp. 1393–1402, 2007.

[57] F. O. Martinez, A. Sica, A. Mantovani, and M. Locati, "Macrophage activation and polarization," *Frontiers in Bioscience*, vol. 13, no. 2, pp. 453–461, 2008.

[58] D. Ruffell, F. Mourkioti, A. Gambardella et al., "A CREB-C/EBPβ cascade induces M2 macrophage-specific gene expression and promotes muscle injury repair," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 41, pp. 17475–17480, 2009.

[59] L. Zhao and R. D. Brinton, "Suppression of proinflammatory cytokines interleukin-1β and tumor necrosis factor-α in astrocytes by a V_1 vasopressin receptor agonist: a cAMP response element-binding protein-dependent mechanism," *The Journal of Neuroscience*, vol. 24, no. 9, pp. 2226–2235, 2004.

[60] M. Sandri, "Signaling in muscle atrophy and hypertrophy," *Physiology*, vol. 23, no. 3, pp. 160–170, 2008.

[61] J. G. Tidball and S. A. Villalta, "Regulatory interactions between muscle and the immune system during muscle regeneration," *American Journal of Physiology: Regulatory Integrative and Comparative Physiology*, vol. 298, no. 5, pp. R1173–R1187, 2010.

[62] J. M. Sacheck, J.-P. Hyatt, A. Raffaello et al., "Rapid disuse and denervation atrophy involve transcriptional changes similar to those of muscle wasting during systemic diseases," *The FASEB Journal*, vol. 21, no. 1, pp. 140–155, 2007.

[63] S. C. Bodine, E. Latres, S. Baumhueter et al., "Identification of ubiquitin ligases required for skeletal Muscle Atrophy," *Science*, vol. 294, no. 5547, pp. 1704–1708, 2001.

[64] M. D. Gomes, S. H. Lecker, R. T. Jagoe, A. Navon, and A. L. Goldberg, "Atrogin-1, a muscle-specific F-box protein highly expressed during muscle atrophy," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 25, pp. 14440–14445, 2001.

[65] A. Smith, R. I. Stephen, M. M. Arkley, and N. McIntosh, "Immunoreactive arginine vasopressin in human fetal and neonatal skeletal muscle," *Early Human Development*, vol. 28, no. 3, pp. 215–222, 1992.

[66] S. L. Alexander, C. H. G. Irvine, M. J. Ellis, and R. A. Donald, "The effect of acute exercise on the secretion of corticotropin-releasing factor, arginine vasopressin, and adrenocorticotropin as measured in pituitary venous blood from the horse," *Endocrinology*, vol. 128, no. 1, pp. 65–72, 1991.

[67] V. A. Convertino, L. C. Keil, E. M. Bernauer, and J. E. Greenleaf, "Plasma volume, osmolality, vasopressin, and renin activity during graded exercise in man," *Journal of Applied Physiology: Respiratory Environmental and Exercise Physiology*, vol. 50, no. 1, pp. 123–128, 1981.

[68] B. Melin, J. P. Eclache, G. Geelen et al., "Plasma AVP, neurophysin, renin activity, and aldosterone during submaximal exercise performed until exhaustion in trained and untrained men," *European Journal of Applied Physiology and Occupational Physiology*, vol. 44, no. 2, pp. 141–151, 1980.

[69] B. Melin, C. Jimenez, G. Savourey et al., "Effects of hydration state on hormonal and renal responses during moderate exercise in the heat," *European Journal of Applied Physiology and Occupational Physiology*, vol. 76, no. 4, pp. 320–327, 1997.

[70] N. de Jager, N. J. Hudson, A. Reverter et al., "Chronic exposure to anabolic steroids induces the muscle expression of oxytocin and a more than fifty fold increase in circulating oxytocin in cattle," *Physiological Genomics*, vol. 43, no. 9, pp. 467–478, 2011.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 818107, 16 pages
http://dx.doi.org/10.1155/2014/818107

Review Article

Influence of Immune Responses in Gene/Stem Cell Therapies for Muscular Dystrophies

Andrea Farini,[1] Clementina Sitzia,[1] Silvia Erratico,[2] Mirella Meregalli,[1] and Yvan Torrente[1]

[1] *Laboratorio Cellule Staminali, Dipartimento di Fisiopatologia Medico-Chirurgica e dei Trapianti, Università degli Studi di Milano, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Centro Dino Ferrari, Via F. Sforza 35, 20122 Milano, Italy*
[2] *Ystem s.r.l., Via Podgora 7, 20122 Milano, Italy*

Correspondence should be addressed to Mirella Meregalli; mirella.meregalli@unimi.it and Yvan Torrente; yvan.torrente@unimi.it

Received 27 January 2014; Revised 7 April 2014; Accepted 30 April 2014; Published 19 May 2014

Academic Editor: Fabio Rossi

Muscular dystrophies (MDs) are a heterogeneous group of diseases, caused by mutations in different components of sarcolemma, extracellular matrix, or enzymes. Inflammation and innate or adaptive immune response activation are prominent features of MDs. Various therapies under development are directed toward rescuing the dystrophic muscle damage using gene transfer or cell therapy. Here we discussed current knowledge about involvement of immune system responses to experimental therapies in MDs.

1. Introduction

The involvement of inflammation in muscular dystrophies (MDs) has been known for years. However, molecular mechanisms underlying immune system activation are not completely understood. Inflammation and innate immune response activation are firstly a consequence of physiological function of skeletal muscle, but their chronic activation is determined by continuous cycles of muscle fibers degeneration/regeneration. MDs are a heterogeneous group of diseases caused by mutations in different components of sarcolemma, extracellular matrix, or enzymes [1]. Despite differences in genetic background and symptoms, MDs share some characteristic features such as progressive muscular wasting, fibrosis and atrophy, and various degrees of inflammatory infiltrates. Here we described the well-known involvement of the cells of the immune system in the development of the pathological signs of the most frequent forms of MDs—Duchenne Muscular Dystrophy (DMD) and dysferlinopathies (LGMD2B)—and the emergent role of these cells in the facioscapulohumeral muscular dystrophy (FSHD). Moreover, we investigated the relationship between immune system and gene or cell therapy in the treatment of these diseases. DMD is characterized by mutations in dystrophin gene: its absence at the sarcolemma reduces the stability of plasmamembrane and renders muscular fibers more prone to contraction-induced injury [1]. In LGMD2B the mechanism of membrane repair is inefficient due to the absence of dysferlin protein, which probably regulates vescicular trafficking [2]. Molecular mechanisms underlying FSHD are not fully understood but it is known that the contraction of a repeated region in chromosome 4q35 leads to toxic activation of DUX4 gene (i.e., normally silenced), which probably acts like a transcription factor [3]. As we discussed below, a certain degree of inflammation is always present in whatever form of MD, so that this condition is probably due to the muscular degeneration itself. However some aspects, such as complement system deposition or specific lymphocytes activation, are typical of one form of MD suggesting a correlation with the genetic background. Finally we discussed how immune system activation could affect gene or cell therapy and how it could be the target of new treatments.

2. Immune System Activation in Skeletal Muscle

In physiological condition, skeletal muscle contains resident immune cells, mainly macrophages, that exert multiple roles such as phagocytosis of cellular debris and microbes, secretion of cytokines and growth factors, antigen-presentation.

Conversely, following pathophysiological stimuli, skeletal muscle is invaded by several immune cells that secrete soluble molecules, affecting the viability and transcriptional activities of regenerative muscle cells. Unfortunately, the complex mechanisms that regulate the interplay among immune system cells and skeletal muscle stem cells and their modulation of muscular regeneration are far from being really understood. In particular innate immunoresponse of the muscle to injury is mediated by Th1 cytokines (that are the cytokines expressed by a particular subset of T helper cells named Th1) which triggers the activation of classic M1 proinflammatory macrophages, which in turn promote the production of prostaglandins, cytokines, and chemokines [4]. Following the early invasion of macrophages/neutrophils, tumor necrosis factor alpha (TNF-α) is highly expressed, activating macrophages to the M1 phenotype, and also inducing the production of other proinflammatory cytokines. Among them, the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) increases proliferation and inhibits differentiation of muscle stem cells. In fact, NF-kB allows the expression of transcripts needed for cell cycle progression and causes destabilization of MyoD mRNA and degradation of MyoD protein, negatively affecting the capacity of muscle to terminal differentiation [5]. Furthermore, recent findings demonstrated that the secretion of the TNF-α in the injury site is necessary for the attraction of satellite cells and, thus, for the promotion of muscle regeneration [6]. In a second time, as M1 macrophages reached the peak of concentration in injured/regenerative muscle, Th2 cytokines (IL-4, IL-10, and IL-13) stimulation promotes a switch toward M2 anti-inflammatory macrophages, which diminish the inflammatory response and promote tissue repair [7, 8]. The transition from a Th1 inflammatory response to a Th2 inflammatory response is closely correlated with a transition from the early proliferative stage of myogenesis (driven by the transcription factors Myod and myf-5) to the terminal stages of myogenesis (driven by Myogenin and MEF2). Interestingly, the functional linkage between M1/M2 differentiation and myogenic compartment was suggested, as the disruption of the Th1 to Th2 transition causes the failure of the transition from proliferative to differentiation stages of myogenesis, in particular at a stage at which satellite cells are activated to proliferate and express MyoD [6]. Similarly, different works demonstrated the fundamental role of M2 macrophages in promoting muscle regeneration, as the depletion of this subpopulation of macrophages prevented increases in muscle fiber diameter and diminished the ability of muscles to repair, to differentiate, and to regenerate [9]. Muscular alterations render the myofibers more vulnerable to contraction-induced injury so that continuous activation of the immune system is present. Chronic inflammation ultimately ends in fibrosis deposition and atrophy, a process mainly mediated by a transition from M2a macrophages to M2c macrophages [10–12]. Contemporary macrophages and myokines secreted by muscle fibres recruit additional immune cells, including T cells, which exacerbate muscular damage. In DMD muscles macrophages are the most abundant immune cells, but T cells, B cells, and dendritic cells (DCs) are also present. Infiltrating T cells are predominantly CD4+, and smaller numbers of cytotoxic CD8+ T cells were observed [13]. In addition, specific T cell receptor gene rearrangements were observed in clonally activated T cells, within dystrophic muscle fibers [14]. Furthermore, recently, Mendell et al. observed specific autoreactive T lymphocytes, directed against dystrophin epitopes, in blood of dystrophic patients (DMD) [15]. Several authors demonstrated that the ablation of T (or T/B cell) cells ameliorated muscular pathology in dystrophic animal models [16–18]. This amelioration was due both to direct ablation of cytotoxic T cells and to reduced secretion of proinflammatory cytokines. Among them, higher levels of TNF-α, IL17, IL6, and TGF-β were observed in DMD muscles than in healthy ones [19]. In particular, TNFα has been suggested to promote necrosis through a regulated process involving kinase activity of receptor-interacting protein 1 (RIP1) [20]. More recently, Burzyn and colleagues described a population of regulatory T (Treg) cells expressing Foxp3 and CD4 that rapidly accumulated in the acutely injured skeletal muscle of mice, similar to myeloid cells. They demonstrated that these cells exerted a fundamental role in regulating muscle repair by the expression of specific growth factors [21]. Immune system activation is not a predominant feature only of DMD; indeed other forms of MDs share the same characteristic, even if different subpopulations of immune cells might be affected. Inflammatory cells were detected in both MM and LGMD2B patients around necrotic fibers. Dysferlin deficiency reduced the capacity of muscle membranes to repair after injury prolonging the recruitment and the activation of inflammatory cells. Enhanced phagocytic activity of dysferlin-deficient macrophages was reported [22] together with components of i(NALP)-3 inflammasome pathway upregulated and activated [23]. Specific deposition of membrane-attack complex (MAC) at the surface of muscle fibers was observed in dysferlin-null mice, demonstrating an important role of the complement factors in exacerbate muscular damage [24]. Skeletal muscle under physiological condition do not constitutively express major histocompatibility complex (MHC) class I molecules: different stimuli however can induce their expression, such as the proinflammatory cytokines IFN-γ and TNF-α [25]. Expression of MHC class I on the surface of muscle cells is an early feature in human idiopathic inflammatory myopathies, also preceding inflammatory infiltrates [26]. It was demonstrated that human skeletal muscle myoblasts, once stimulated to express MHC class I or even class II, can effectively present antigens to autologous, antigen-specific CD4+ T cells [27]. In dysferlinopathies abnormal MHC-I expression was observed in degenerating/regenerating fibers usually closed to inflammatory cluster cells. A direct pathogenic role for immunity has never been demonstrated in FSHD. Inflammatory features were evident in biopsies from FSHD patients [28, 29], whereas a pilot trial involving prednisone did not improve strength in FSHD muscles [30]. More recently, magnetic resonance imaging (MRI) was used as powerful method to identify specific areas of the muscles in different disorders [31]. This way, Tasca et al. demonstrated a correlation between hyperintensity in T2-STIR sequences and histological muscle abnormalities and showed that these areas were characterized by inflammation [32]. Moreover, to better elucidate the possible pathological

mechanisms underlying inflammatory and immunological processes, they studied the amount of activated immune cells in the blood of FSHD patients and their expression of cytokines and other immune regulators. They found a significant amount of CD8+ T-cells in the endomysium of FSHD muscles, close to nonnecrotic fibers. They also noted that FSHD patients overexpressed pSTAT1 (regulator of Th1 cells), pSTAT3 (regulator of Th17 cells), and t-bet (regulator of both innate and adaptive immunity) that cause overproduction of IL12/IL23p40, IFN-γ, TNF-α, IL6, and IL10 related to controls [33]. The excessive secretion of IL6 driven by monocytes probably caused CD8+ T cell activation, allowing uncontrolled proliferation and effector functions. Interestingly, they did not assess any modification of the complement system as suggested in the dysferlinopathies [34]. A study published in 2012 confirmed that in the blood of FSHD patients several genes that mediated innate and adaptive immune response were upregulated, assessing that inflammation had a central role in the development of pathological phenotype [32].

3. Inflammation-Based Therapeutic Strategies

Despite all efforts exerted, steroids are the only current treatment available for DMD. The exact mechanism of action of steroids in DMD patient is not fully characterized but the beneficial effects observed are probably due to their immunosuppressor ability. For example, it is thought that glucocorticoids promote a shift from M1 to M2 anti-inflammatory macrophages, as observed in patients treated with prednisone (0.75 mg/kg/day) during 6 months [35]. As we discussed above, steroids treatments are also associated with lower levels of autoreactive T-lymphocytes in DMD patients [36]. The involvement of cytokines and other inflammatory actors in MDs opened new therapeutic strategies. In particular, modulation of proinflammatory cytokines, such as TNFα, is currently under investigations. Infliximab and Etanercept (TNFα neutralizing antibody and soluble TNFα receptor, resp.) both showed reduction in fibrosis deposition and in myofiber necrosis in mdx mice [37, 38]. Similarly, blocking chemokine-mediated signalling, through chemokine-receptor antibodies (including an anti-CCR2) seemed to be promising. Major concerns with this strategy involved the abundance of chemokines that are upregulated in DMD patients and the interpatient variability. Furthermore, as we discussed above, chemokines are necessary to attract non-cytotoxic macrophage subpopulations, which are responsible for myofibers regeneration [9]. In the same way TGF-β blocker suramin and the TGF-β1 antagonist pirfenidone decreased fibrosis in mdx mice [39, 40]. Immunomodulatory strategies have been investigated in other forms of MDs such as dysferlinopathies. Unfortunately, anti-inflammatory glucocorticoids gave negative results in treated patients probably because of side effects [41, 42]. However, alternative strategies, such as intravenous immunoglobulins and Rituximab administration, showed partial benefits in LGMD2B patients although B lymphocytes are not a major component of infiltrates in this disease [43]. Similar to what observed in DMD patients, the administration of Etanercept resulted in dose-dependent reductions of inflammation, necrosis, and fatty/fibrous change in dysferlinopathic SJL/J mice [44]. Recently, Halofuginone, a coccidiostat (T helper 17 cells inhibitor), was shown to significantly improve dystrophic features in a dysferlin-deficient mouse model [45]. As discussed above, complement system plays a central role in promoting myofiber necrosis in dysferlinopathies, so that it represents an attractive target for therapies. In this sense genetic ablation of C3 improved muscle phenotype in dysferlin-deficient mice [24]. To date, immunomodulatory therapies for FSHD, including a prednisone pilot trial, failed to demonstrate beneficial effects [30].

4. Influence of Immune Responses in Gene Therapy

Gene therapy is commonly used to correct or to replace genes whose mutations cause fatal disease. It is characterized by 3 critical steps: the genes that are transferred, the target tissue, and the carrier of the gene. The carrier has to allow the entry of the gene into damaged tissues: retrovirus, lentivirus, adenovirus, and adenoassociated virus (AAV) are commonly used as vectors. AAVs can infect long-lived postmitotic cell; their DNA can be integrated into the host cell's genome and donated gene is long-term expressed [46]. As the administration of such viral vectors could cause host immune response, clinical protocols for gene therapy require that sustained therapeutic levels of the transgene are achieved, with no apparent vector-related toxicities in the patient [47, 48]. In this section, we will describe the effects of administration of AAVs in the most common forms of neuromuscular disorders.

4.1. Adeno Associated Virus (AAVs) and Immune Reactivity. AAVs are nonpathogenic and replication-defective—as they did not possess any viral protein ORFs—that can infect nondividing cells. To date, 12 different AAV serotypes (AAV1-12) are utilized in gene therapy approaches, regarding liver (AAV8), cardiac, and musculoskeletal tissues (AAV1, 6 and 9), central nervous system (AAV5 and 9), and eye (AAV4 and 8) [47]. AAVs have low immunogenicity compared to adenovirus as they did not transduce efficiently macrophages, mature DCs, and other antigen-presenting cells (APCs); however, different clinical trials demonstrated that these vectors can cause immunological responses [49]. Furthermore, it is known that AAVs are nonintegrating vectors but insertional mutagenesis could occur [50]. Zaiss and Muruve showed that APCs can take up exogenous AAV antigenic peptides by endocytosis; consequently, AAVs are released in the endosomes, entering into the nucleus and viral genome induces uncontrolled gene expression [51]. This way, innate immune signalling pathways are triggered and the secretion of several factors that regulate inflammatory response allows the expression of proinflammatory cytokines and chemokines [52, 53]. All these molecules favour the infiltration of neutrophils, macrophages, and DCs that kill the transduced cells directly or initiate a more specific

T- and B-cell response [54]. Alternatively, it was demonstrated that the complement system—a component of the innate immune system that promotes inflammation [55]—had an important role in the host response to AAV vectors [56], as their interaction caused the activation of macrophages against AAVs [56]. Other than innate immunity, AAVs could be identified and destroyed by the adaptive immune responses. Once the vector is brought inside the host cells, the viral capsid is degraded so that antigenic peptides are cross-presented to MHC I molecules and CD8+ T cells destroy the transduced host cell [57]. Activated CD8+ T cells can elicit indirectly the function of CD4+ T cells by secreting proinflammatory cytokines (IFNγ, TNF-α, and IL-2) [57]. Alternatively, APCs could present the AAVs to T-cell receptor (TCR) of CD4+ T cells by means of MHCII complex [58]; this way, CD4+ T cells allow the expression of proinflammatory cytokines (IL-2, IL-4, and IL-5) that can activate B cells [59]. At this point, specific antibodies against the particles of AAVs are produced and then eliminated by neutrophils and DCs [59]. As eyes are considered ideal organs for AAVs infusion for their immune-privileged status, the works of Maguire et al. [60] and Hauswirth et al. [61] demonstrated that the ocular injections of AAV vector are a safe and efficacious technique to ameliorate visual function in LCA patients. For all the other organs, immunosuppression protocols were proposed to reduce or prevent host immune response following AAVs injection. In the case of degenerative disorders such as haemophilia and muscular dystrophies, continuous administration of AAVs is required for therapeutic purposes. Unfortunately, secondary exposure to the vector strengthens the risk of activation of memory T- and B-cells, immune reactivity events, and vector elimination. It was suggested that these problems could be overcome with short-term immune suppression treatment in the initial phase of vector infusion [62, 63]. In addition to immunological questions, the other milestone of gene therapy is its translation into large animals rather than mice. To this end, Valentine et al. used the beagle-based canine X-linked muscular dystrophy (CXMD) which shared with DMD patients several pathological features [64]. Following injection of rAAV2 driven by muscle-specific promoter, they described a significant infiltration of cells 2 weeks after the treatment. In particular they identified CD4+ or CD8+ T lymphocytes in the interstitial spaces of the injected muscle while CD11b+ cells and B cells were found in clusters among infiltrating cells. More interestingly, muscle fibers upregulated MHC classes I and II molecules [65]. Experiments of administration of the rAAV expressing no transgene into the CXMD muscles suggested that the strong immune response that takes place in treated muscles and that causes the elimination of transduced myofibers is due to transgene product and not to AAV capsid [65].

4.2. AAV Treatment in Neuromuscular Disorders. Adriouch et al. described a strategy to inhibit the undesirable immune activation that follows muscle gene transfer. The cytotoxic T-Lymphocyte Antigen 4 (CTLA-4 or CD152)—a protein receptor that downregulates the immune system that is present on the surface of T cells, leading the cellular immune attack on antigens—was used to block the costimulatory signals that are required early during immune priming. In combination with the supplementation of programmed cell death protein 1 (PD-1) to inhibit T cell functions at the tissue sites, they efficiently modulated the immune response of AAVs transduced muscle cells [66]. Similarly, Lorain et al. characterized the immune response to the AAV1 vector in the DMD murine model, the mdx mouse. They explored methods to block interactions between T cells, antigen-presenting cells and B-cells by interfering with the costimulatory pathways B7/CD28 (CTLA4) and CD40L/CD40, using two specific agents, CTLA4/Fc and MR1. They demonstrated that this immunomodulatory treatment completely blocked the formation of antibodies against the AAV1 vector and enhanced the expression of dystrophin in several muscles [67]. Mendell and coworkers showed that LMGD patients injected with AAV1 had both humoral and cellular immunity against the capsid even if the expression of the transgene was not impaired by the immune response [68]. To avoid the recognition of AAVs by T cells and to ameliorate the efficiency of transduction, the immunogenic epitopes of the capsid were masked. Furthermore, tissue-specific promoters were used to modulate the expression of the transgene only in the target tissues, avoiding the recognition by APCs and subsequent activation of the immune system [54].

4.2.1. Gene Therapy and DMD. According to their low immunogenicity coupled with their "cargo" capacity, AAVs were largely used to treat fatal neuromuscular disorders. Although the genome of AAV persists into muscle for several years, it was demonstrated that the integration of the AAV genome into host myogenic cells was largely inefficient and disappeared rapidly with time. Schnepp et al. suggested that very little rAAV vector DNA integrated in transduced mouse muscle and that viral DNA persisted only as concatameric episomes [69]. Despite these problems, different serotypes of AAV were used with encouraging results in model mice of DMD. rAAV serotypes 6 and 8 efficiently delivered the microdystrophin cassette to skeletal muscle in the mdx mouse through the vasculature [70]. Moreover, they achieved skeletal muscle transduction also in compartments far from the site of injection, such as limb muscles [70]. Similarly, Wang et al. demonstrated that AAV8 is the most efficient vector for crossing the blood vessel barrier and AAV8-mediated gene expression persisted in muscle and heart, but diminished in tissues undergoing rapid cell division [71]. As direct injection of rAAV1 was efficient and safe [69], this vector was transplanted into femoral artery of mdx mice, allowing robustly reexpression of dystrophin [72]. However, it was showed that the capacity of transduction of rAAV6 and 8 was significantly better than rAAV1, concerning the amelioration of isometric force in treated mice [73]. Another important problem in the treatment of DMD patients arose for their possible immune response to vectors-carried dystrophin. The risk of T-cell immunity can be strongly reduced whether the differences between the defective self-gene and the therapeutic transgene are limited. It could be the case of pathologies that are provoked by a small number of missense mutations

but the pathologies that are caused by large genomic deletion of specific genes (such as dystrophin in DMD and BMD) increased the risk of this kind of immunological reaction. In fact the protein in DMD/BMD patients is absent or present in abnormal form so that gene transfer mediated by viral vectors could cause the development of immune responses to previously unseen epitopes. As a confirmation, antibodies specific to the donor dystrophin were seen in BMD and DMD patients that received, respectively, cardiac [74] and donor myoblast transplantation [75, 76]. To avoid that other proteins introduced with dystrophin in these experiments could alter the specific immune response to dystrophin, Ferrer et al. injected naked plasmid DNA into skeletal muscle of mdx mice to be sure that no potential neoantigens were introduced and described a specific immune responses to dystrophin [77]. Although the first DMD trials based on myoblast transplantation showed a partial expression of dystrophin [76, 78, 79], they failed probably for immunological reaction of these patients to dystrophin. Ferrer et al. demonstrated that, following injection into mdx mice of plasmid encoding for minidystrophin, the newly formed dystrophin positive myofibers were destructed four week after the treatment through a cell-mediated immune response. In particular, cytotoxic CD8+ T cells were identified in clusters around the dystrophin + fibres in the injected leg [77]. Interestingly, they also noted that full-length dystrophin was less immunogenic than the minidystrophin, probably for the presence of suppressor epitopes in the rod domain that is deleted in the minidystrophin [77]. Similarly, Yuasa et al. treated mdx mice modified to express minidystrophin with AAVs; they showed that immune responses were mediated by the membrane permeability and assessed that, using muscle-specific promoters, the activation of the immune system was significantly delayed [80]. Starting from data from DMD clinical trials that confirmed how the therapy with prednisone ameliorated the pathological phenotype and strength of muscle [81], they suggested that immunosuppression not only ameliorated the efficiency of transduction mediated by the AAVs but above all limited the degeneration of myofibers blocking the immunological cells that recognize and destroy newly formed dystrophin positive myofibers [80]. More recently, Mendell and coworkers treated a group of six DMD patients with AAV carrying a minidystrophin transgene that partially restores the generation of muscle force in dystrophic mice [15]. As expected, following the treatment, they identified dystrophin-specific T cells but, surprisingly, circulating dystrophin-specific T cells were found in two patients before AAV treatment. Furthermore, they assessed that autoreactive T cells recognized epitopes that were presented on revertant dystrophin fibers. This way, they suggested that to increase the efficiency of experimental therapy for DMD in term of formation of dystrophin positive myofibers, T-cell immunity to self and nonself dystrophin epitopes has to be accurately investigated [15]. Recently, Flanigan et al. evaluated dystrophin-specific T cell immunity in DMD patients that were treated with glucocorticoid steroids [82]. They showed that not only the risk for the presence of antidystrophin T cell immunity increased with age but, more interestingly, steroid-treated patients developed milder immune response related to not treated patients. Steroids could exert their beneficial effects in DMD patients by modulating T cell responses [82]. In addition to AAVs, HIV-derived lentiviral vectors were used to transfer genes to muscle; they can infect both dividing and nondividing cells and possess the capacity to clone minidystrophin together with selectable markers and the promoters needed for transgene expression [83]. These vectors permanently transduce and stably express transgenes in muscle cells (and their precursors) [84, 85]. Unfortunately, their use in clinical approaches was largely restrained for safety reasons. HIV could self-replicate and produced during manufacture of the vector in the packaging cell line or in the target cells by a process of recombination; consequently, the patients undergoing gene therapy would be infected with HIV in addition to the new therapeutic gene. Moreover, self-replicating infectious vector could cause uncontrolled insertion into host genome and activate prooncogenes causing cancer [86].

4.2.2. Gene Therapy Applicability in LGMD and FSHD. Differently from dystrophin, the full-length cDNA of each isoform of the sarcoglycans (whose mutations cause the LGMD) can be cloned into the AAV so that AAV-based gene therapy is feasible for this disease. In different studies, Li and Greelish demonstrated the feasibility of AAV-mediated gene transfer of δ-sarcoglycan into skeletal muscle using cardiomyopathic hamster [87, 88] but this model—as the majority of the murine ones—did not face the pathological condition of LGMD muscles. In fact, the rate of ongoing degeneration-regeneration cycles is too slow so that muscle maintains its function and fibrosis did not develop well. This way, Hack et al. developed a new murine model for primary γ-sarcoglycan deficiency that exhibited the clinical and histopathological characteristics of LGMD: degeneration and regeneration of muscles, pseudohypertrophy, and development of fibrosis. All of these features became evident as early as 4 weeks, following muscle membrane disruption [89]. Cordier et al. used this model to test the efficacy of AAV-mediated transfer of γ-sarcoglycan; moreover, to avoid possible toxicity and immunogenicity in nontarget tissues, they used muscle-specific promoter to express the gene only into differentiated muscle [90]. They found that delivery of AAV allowed long-term correction of the disease phenotype and, more interestingly, fibrosis presented a significant barrier for viral delivery. As fibrosis is the last of a series of events that are initiated by the recruitment of immune cells and APCs into muscle to counteract the presence of AAV and its transgene, they suggested that gene therapy has to be performed early in the life of the patients, before that fibrosis occurs [90]. It is now known that FSHD is caused by gene overexpression [91]. The group of Gabellini developed a murine model of FSHD over-expressing the FRG-1 gene that resembled histological and molecular features typical of the disease [3]. Due to its genetic features, FSHD provides a valuable model to test therapeutic potentials of RNAi-mediated gene silencing. Bortolanza et al. combined the delivery mediated by rAAV6 with RNAi-mediated mRNA knockdown and injected rAAV6 expressing FRG1 shRNAs into the tail vein of FRG1 animals [92].

They demonstrated that the treatment was safe and allowed the long-term knockdown of FRG1. Furthermore, the rAAV6 injection ameliorated the pathological phenotype of mice; in particular, the complete functional recovery of the muscular functions was obtained only with the higher dose of vectors. In this contest they did not assess any problem of immune reaction against the product of the vector but they observed that the expression of RNAi cassette could be toxic [92]. As this effect was observed in other diseases [93, 94], it was determined that toxicity was abolished as soon as RNAi hairpin sequences were captured by naturally occurring miRNA scaffold [93].

5. Cell Therapy and Immune Responses

Cell therapies have gained increased attention in the last years. Therapeutic cells could be obtained from patient, corrected *ex vivo,* and retransplanted (autologous implantation). Alternatively, the cells could be isolated from healthy donors and injected into dystrophic patient (allogeneic implantation). The most suitable cells for therapeutic purposes should be easily isolated and retain the capacity to migrate from blood to muscle and to enter the satellite cells niche. Once the host cells fuse, affected muscles will repopulate improving muscle function and pathology. Stem cells were showed both to replenish their numbers for long periods through cell division and to efficiently produce a progeny, differentiating into multiple cell lineages [95]. Embryonic and adult stem cells differ significantly with regard to their differentiation potential and *in vitro* expansion capability. Adult stem cells constitute a reservoir for tissue regeneration throughout the adult life; they are tissue-specific and possess limited capacity to be expanded *ex vivo*. Conversely, embryonic stem cells (ESC) are pluripotent cells derived from the early embryo; they are capable to proliferate over prolonged periods of culture, to remain undifferentiated, and to maintain a stable karyotype [96–98]. In the next section, we will examine the studies in which these cells are involved.

5.1. Immunogenicity of Embryonic Stem Cells and Induced Pluripotent Stem Cells. Different works described good results of engraftment following the injection of ESCs into recipient muscle [99, 100]. Although ESCs could represent reliable and cost-effective therapeutic substitute for treatment of neuromuscular disorders, their transplantation often causes teratomas so that all undifferentiated cells need to be removed from a graft. Taken together with ethical problems in the destruction of the blastocyst, the employment of ESCs in a clinical perspective is far from occurring. In 2006, the group of Yamanaka obtained ES-like induced pluripotent stem cells (iPSCs) from adult mouse and human cells by introducing specific sets of genes encoding for transcription factors expressed in undifferentiated ES cells to reprogram the adult cells [101]. Similar to ESCs, iPSCs retained the ability to differentiate into all adult cell types and, more importantly, their generation does not imply the use of embryonic or foetal material. However the safety of these cells had to be tested accurately before attempting any therapies [101]. Since ESCs were discovered, they were thought to be immune-privileged as their low expression of MHC class I, MHC class II and, conversely, high expression of immunomodulatory molecules regulating the proliferation of T-cell [102]. Unfortunately, it was demonstrated that ESCs allowed donor-specific immune response in immunocompetent mice [103]. The findings of iPSCs opened new possibilities to solve the problem of immune rejection but some hurdles remained. Very preliminary study described that iPSCs were rejected even in MHC-matched recipients, due to unnatural expression of genes that were recognized by CD4+ and CD8+ T cells [104]. Mullally and Ritz suggested that genomic alterations acquired during iPSCs formation/proliferation generated immunogenic neoantigens, potentially eliciting immune responses even in a MHC-matched context [105]. Their observations were confirmed by another work, showing that iPSCs had uncontrolled differentiation capacity due to duplications on chromosome 12 [106]. Furthermore, several studies demonstrated that ESCs rejection was accelerated during upregulation of MHC [103, 107, 108] so that it was suggested that ESCs/iPSCs transplantation could be more difficult into such an environment. Other studies focused on minor histocompatibility antigens (miHA) and determined that even identical HLA phenotype could not be sufficient to guarantee graft survival [109, 110]; in addition, expression of Oct-4 and other specific factors could enhance miHA incompatibilities [111]. It is demonstrated that immunonological problems following cellular injection can be at least diminished by eliminating APCs from the graft before transplantation [112]. As endothelial cells are able to mediate the direct pathway of allorecognition [113], the use of ESCs and iPSCs derived from endothelial cells needs further precautions.

5.2. Immunogenicity of Myogenic Stem Cells. Initial efforts were focused on the progenitor cell in skeletal muscle, the satellite cell, and the descendants of activated satellite cells, the myoblasts. Partridge's group demonstrated that transplantation of wild-type syngeneic myoblasts restored dystrophin expression in immunodeficient mdx mice [114]. Although the first DMD trials based on myoblast transplantation showed a partial expression of dystrophin [76, 78, 79], they failed for donor myoblast survival, as they undergo rapid and massive death after injection into host muscle. It was suggested that host immune response was responsible for the death of the transplanted cells. In particular, taking into account the rapidity with which donor myoblasts die following transplantation, it was thought that the complement system allowed the generation of a membrane attack complex (MAC) through the expression of a C3 convertase, lysing targeted cells quickly. In case of complement depletion, the survival of donor myoblasts was not enhanced so that it was argued that complement could induce the activity of neutrophils and macrophages, indirectly regulating the death of transplanted cells [115]. Different studies demonstrated that myoblast could be recognized directly by host T cells due to the expression of MHC antigens that are recognized by TCR on T cells. Alternatively, myoblasts could be identified

by APCs that present donor antigens to host T-cells [116, 117]. Furthermore, it was supposed that muscle-resident mast cells that are elevated in DMD patients [118] secreted cytokines such as TNF-α, affecting donor myoblast survival [119]. Data obtained by these studies lead to the conclusion that immune suppression was necessary to permit allogeneic myoblasts transplantation [76]. Several studies were conducted using different immunosuppressive protocols, in attempt to avoid side effects of systemic immunosuppression while permitting donor myoblasts survival. Unfortunately none of these studies reported significant or long-term improvement in muscle strength [120–123]. It was thought that stem cells, differently from myoblasts, were immune-privileged so that the risk for immunorejection was underestimated. Today the immunogenicity of stem cells has been widely demonstrated both for embryonic and adult cells [111, 124, 125]. In particular autologous stem cells can provoke inflammation and rejection maybe as a consequence of genetic manipulation or long-term culture (required for their correction and expansion) or their combination with matrix structures. Similarly, ESCs express low levels of HLA class I but only before differentiation; once injected and after reaching the host organ, the MHC disparities lead to acute antibody-mediated rejection (AMR) [126]. Furthermore if IgG antibodies directed against HLA class I antigen of donor cells are already present at the time of transplantation hyperacute rejection (HAR) will occur [127, 128]. Interestingly evidences arose that HLA/MHC molecules could have a role in signalling transduction so that the prediction of efficacy of transplantation is further reduced. MHC expression in transplanted cells could be influenced by the host microenvironment (e.g., INF-γ or hypoxia exposure) [126]. Anti-HLA antibodies mediated injury through both complement-dependent and independent pathways and through the binding with HLA donor cells; this binding could result in activation, proliferation, and cytokines production, leading to amplification of damage [129]. Interestingly, HLA signalling is not a prerogative of immune cells alone, as it also occurs in endothelial or epithelial cells with unknown side effects [130, 131]. Other than satellite cells and myoblast, muscular and nonmuscular multilineage stem cells able to actively participate in myogenesis were identified and characterized according to the expression of different cellular markers [132–138]. However, promising results obtained with animal models were not replicated in humans. In addition, only some of those cells migrated through the vasculature (CD133+, mesoangioblasts, and mesenchymal stem cells) [133, 137, 139, 140] and, this way, were considered for therapeutic interventions. In 2009, Cossu's group started a clinical study in DMD patients ranging in age from 5 to 12 years. The ongoing clinical trial considered intra-arterial infusion of donor mesoangioblasts HLA-identical donor derived in DMD patients pretreated with Tacrolimus. Results of the immune-reaction in this study are in progress.

5.3. Immunomodulation Behaviour of Mesenchymal Stem Cells. Mesenchymal stem cells (MSCs) are currently considered as one of the most promising cell types for cell therapy. Firstly isolated from bone marrow [141], and nowadays obtained from a variety of tissues [142, 143], they are a heterogeneous cell population characterized through different culture conditions and surface markers expression. Apart from the multipotency of cell differentiation, MSCs have been shown to modulate endogenous tissue by secreting a large spectrum of bioactive molecules [144] that were demonstrated to induce different responses, such as angiogenesis [145], inflammatory inhibition, immune modulation, and apoptosis reduction [146]. Furthermore, it is well known that MSCs are immunoprivileged due to their low expression of MHC-II and costimulatory molecules in their cell surface, making them invisible to immune system. The mechanism of action underlying this behaviour involves different immune cell types, both from innate and adaptive immune response. As regards the first type of response, for example, MSCs are able to prevent the two phases of DCs maturation—from precursors to immature state (iDC) [62] and the complete maturation [147, 148]. In both cases MSCs generate a tolerogenic fate of DCs and a possible switch from Th1 to Th2 response [149]. On the other hand, as regards the natural killer cells (NKs), MSCs can prevent their activation and consequent massive release of IFN-γ and TNF-α, with resulting cytotoxic effect [150, 151]. Only if NKs are not previously activated by IL-15 or IL-2, MSCs act as block for NKs lytic ability [152]; otherwise, NKs are able to kill allogeneic and autogenic MSCs, in a process mediated by specific molecules [150, 152]. In the prospective of an application of MSCs in cell therapy, it has to be taken into consideration the possibility that the activation of NKs, due to infections or tumor cells, could interfere to the therapeutic effects of transplanted MSCs. On the contrary, the presence of important concentrations of MSCs could also turn off the innate immune responses of NKs against a future infection or neoplastic development [153]. As regards the adaptive immune response, aspects such as proliferation, differentiation, and maturation of B-cells and their antibody production can be affected by the presence of MSCs in a dose-dependent way [154–157]. MSCs immunosuppressive mechanism is based principally on B lymphocyte-induced maturation protein-1 (Blimp-1) blocking, whose expression is necessary for Ig production [154, 156]. Also T-cell responses are affected by MSCs properties; proliferation, release of IFN-γ, and cytotoxicity are influenced in a dose-dependent manner [158]. All these effects seem to be mediated by the release of soluble factors, such as TGF-β and IL-10; in addition, the nitric oxide (NO) should play a major role in the MSC-mediated T-cell suppression, through a mechanism of inhibition of signal transducer and activator of transcription 5 (STAT5) phosphorylation that prevents T-cells from entering the cell cycle [159–161]. In both cases (regulation of B- or T-cells), the MSCs need to be previously activated to exert their suppressive properties. In a synergic view of immunosuppressive behaviour, MSCs increase the proliferation of T-regs, immune cells capable of suppressing the proliferation of activated T cells, in order to avoid a host damage due to an exaggerated immune response. In fact, it has been demonstrated *in vitro* that when MSCs are added to a mixed lymphocyte reaction the percentage of T-reg

increases [147], while *in vivo*, the same result is reached after the injection of MSCs intravenously [162]. Several studies demonstrated the ability of MSCs to diffuse in dystrophic host muscles once injected in utero- or intravenously in mdx mice together with some dystrophin reexpression [163–165]. Although these studies confirmed the possibility of MSCs transplantation without immunosuppression, they did not report any increase in muscle strength [166, 167]. Recently Vieira et al. demonstrated that human adipose-derived mesenchymal stromal cells were able, once injected systemically into GRMD dogs, to reach the host muscle and to express human dystrophin without any need of immunosuppression [168]. These data are the starting point for the development of new therapeutic strategies.

6. Conclusions

In MDs the chronic progression of the disease leads to exhaustion of muscle regenerative potential, so that gene therapy will result to be useless without supplying new muscular fibres. In this point of view cell therapy represents the best tool to deliver both the functional gene and the myogenic potential required. Gene therapy is focused on replacing the defective gene with a new one carried by different vectors; AAVs-mediated gene therapy for MDs is a feasible therapeutic approach that leads to the planning and implementation of phase I clinical trials. Initially on preclinical studies, the treatment with AAVs seemed to be minimally immunogenic, but data from limited human trials evidenced the concept of vector dose-dependent immunotoxicity [169, 170]. Moreover, transgene cassette and capsid structure have been shown to stimulate an immune response [171, 172]. As it became evident that unique AAV serotype will not be universally applicable for therapeutic gene transfer, other naturally occurring alternate AAV serotypes have to be developed and utilized [173, 174]. Moreover, modifications of these vectors could increase their transduction efficiency and consequently reduce dose-dependent immune response. To guarantee a relevant restoration of muscle function in DMD patients, it is necessary to perform repetitive AAV infusions to target multiple muscular territories. Many works showed the decrease of the efficiency of gene transfer after repeated injections of the AAV2 capsid in normal mice. Unfortunately, there is no animal model which can accurately predict the anti-AAV immune response in humans [59]. MDs, despite their heterogeneity, share some common features like progressive muscular weakness, atrophy, and inflammation. Furthermore, chronic injury determines *per se* immune system activation. The immune system response has different components: the humoral immunity, the cell mediated immunity, and the inflammatory pathway that includes the complement system and the macrophages or NKs activation. All these components are involved at different levels in each MDs form and they are differently activated against cell- or gene-therapy both in a specific manner, linked to the type of cell or vector used, and according to MDs-specific immune-pathogenetic mechanisms.

A new emerging point is the regulation of genes involved in immune response which are overrepresented in human population, so that some pathways are subjected to positive or balancing selection [175, 176]; in particular, they were identified genes related to cytokines (IL-1 receptor agonists) and MHCI-related antigen presentation (as TAP1) [177–179]. These genetic variants are responsible for phenotypic diversity and adaptation against viral/bacterial infection but also to susceptibility to autoimmune diseases. The genetic predisposition could be involved both in variability of response to treatment (as differences among DMD patients in steroid's treatment response) and in immunity response against gene therapy. In fact the ability to present antigen with MHC-I molecules could influence the number of antidystrophin CD4+ T cells produced after AAV injection in DMD patients. In the future, patients could be characterized for their genetic predisposition to develop an immune response as a tool for patient risk stratification and to administrate high-dose of immunosuppression only if it is indispensable.

Humoral Immunity in MDs. Partial benefits were observed in LGMD2B patients with intravenous immunoglobulins and Rituximab (anti-CD20) administration; both of these treatments blocked B-lymphocytes which are not a major component of infiltrates in this disease [43], suggesting that further studies are needed to clarify the role of B-lymphocytes in this muscle disease. Among alternative strategies to bypass the problem of immune response, the immunomodulatory effect of intravenous immunoglobulin (IVIG) was used in immune-mediated diseases such as multiple sclerosis and myasthenia gravis [180, 181] and, largely, in neuromuscular diseases [182]. It was postulated that the IVIG in association with cell and gene therapy could modulate the immune system via multiple putative mechanisms. Therefore, as a theoretical rationale for the use of IVIG treatment was demonstrated for inflammatory diseases [183], the specific effect in MDs has to be completely understood. Lorain et al. described the humoral immune response AAVs following intramuscular injection of AAV1-U7. As it could preclude the success of subsequent AAV1 infusions if administered more than 3 days after the primary injection, they eliminated this adverse immunity by means of CTLA4/Fc and MR1, which are currently being evaluated in human clinical trials [67]. CTLA4/Fc has been used successfully in animal models of autoimmunity [184] and transplantation [185] and has been approved by the Food and Drug Administration for clinical use. Other studies showed that AAV vectors may induce humoral adaptive response to AAV *in vivo* as the vectors interact with different complement components [186]. Therefore, these results indicated that it is necessary to combine gene therapy with immunosuppressive or immune-modulant therapy to prevent immune system activation and to allow the expression of the transgene.

Cell Mediate Immunity in MDs. In DMD a self-directed T cell activation is emerging as the major obstacle to gene or cell therapy as T-CD4+ cells directed against dystrophin protein and not against exogenous vector or donor cells

were observed [15]. This evidence suggests that immunosuppressive therapy should be coupled with other preventive strategies to induce persistent antigen-specific tolerance in the gene therapy setting [187]. This cell-mediated autoimmune component was never observed in dysferlinopathies, where altered macrophages function, complement deposition, and cytokines release are the major features [22]. Only poor data are available on FSHD pathophysiology so that specific immune mechanism is not yet described. Steroid treatment is the only therapeutic strategy available for DMD. Interestingly Flanigan et al. demonstrated that steroids also reduced the number of patients' autoreactive antidystrophin T-cells [82]; accordingly, steroid treatment could be used as immunomodulator together or before starting a gene or cell therapy to facilitate the survivor of dystrophin expressing fibers. Unfortunately both in dysferlinopathies and in FSHD, steroids' clinical trials failed to ameliorate or delay the disease progression [30]. In case of DMD, cell therapy focused on administration of dystrophin-expressing myoblasts. Tremblay et al. transplanted myoblasts in 5 patients without cyclosporine. Unfortunately, no increase in isometric force was observed and, 6 months after the injection, less than 1.5% of dystrophin-positive fibers were found. This study demonstrated that immune suppression was necessary for the transplantation of allogeneic myoblast [76]. However, in 1993, Karpati et al. showed that no functional improvement or dystrophin expression were found after transplantation of 55 million of myoblasts in the biceps of 8 DMD patients under cyclophosphamide immunosuppressive treatment [120]. Since the problem arose with myoblast transplantation, many research groups focused their attention on stem cells but the risk for immunorejection was underestimated. In fact ESCs once differentiated in the host tissues' ability to lose their "immune-privilege state" thanks to MHC class I expression, favoured by host microenvironment [126]. Similarly IPSCs can be recognized by CD4+ and CD8+ T-cells through unnatural-manipulated genes and also autologous stem cells can be modified by long-term culture or supports materials [111, 124, 125]. As we stated before, the immunomodulatory properties of MSCs are so appealing to consider these cells for clinical purposes. Many studies demonstrated that the therapeutic effects of MSC are due not only to direct differentiation into injured tissue but also to production of paracrine factors that are able to inhibit apoptosis, to increase endogenous cell proliferation, and/or to stimulate tissue resident stem cells in the site of injury [188]. Together with MSCs, several stem cell populations (resident and nonresident in muscle) were investigated for their ability to ameliorate the pathological phenotypes of muscular dystrophies. Among them, some not only were differentiated into muscle but, more importantly for the treatment of devastating muscle disease, retained the ability to migrate through vasculature and reach all the muscles of the body. Our group demonstrated that CD133+ stem cells isolated from muscle can be injected safely into DMD patients [189] and, genetically modified, improved muscle function and allowed dystrophin expression following transplantation into dystrophic mice [133]. According to these results, we will start a clinical trial where we treat DMD patients with their own CD133+ stem cells *ex vivo* engineered with a lentiviral vector carrying the AONs sequences able to skip the exon 51. Similarly, very promising results were obtained by injecting muscle-derived stem cells (MDSCs) [190, 191] and mesoangioblasts [192–194] into dystrophic animal models. An ongoing clinical trial, promoted by Cossu's group, will assess the feasibility of intra-arterial transplantation of mesoangioblasts, from HLA-identical donors, in DMD children treated with bland immune suppression.

In the last years, many ways were undertaken in order to find the best conditions to decrease the host immune reaction after gene or cell therapy. In 2013, Figueiredo et al. demonstrated that MHC class I silencing significantly prolongs cell survival after allogeneic transplantation by preventing the identification from the immune system. Silencing MHC expression on transplanted cells could represent a potential field able to revolutionize the cell-based products developed for regenerative medicine and particularly for muscular dystrophies treatment [195].

Inflammatory Pathway in MDs. During tissue regeneration events, infiltrating inflammatory cells and resident cells interacts precisely. Impairment of these events can cause unsuccessful regeneration and develop a condition of injury, typical of the MDs (as described above in the Section 2 "Immune system activation in skeletal muscle"). To defeat inflammation is one of the most important goals of clinical experimentations, as transplantation of cells into such an environment limited dramatically their survival, due to activation of macrophages.

Steroids are efficient not only in modulating cell-mediated immunity but also in attenuating the inflammatory pathways involved in MDs. As they are associated with important adverse effects, it could be important to better elucidate the factors that drive inflammation to obtain more selective immunomodulatory intervention. As discussed above, impairment in dysferlin-mediated membrane repair promotes a destructive inflammatory response by activating the innate immune system. However, all the pharmacological treatments tested failed to work in dysferlin-deficient patients [41, 42]. To date, encouraging results were obtained by studying the NLRP3 inflammasome signalling pathway so that it is now considered as a good therapeutic target for dysferlinopathy [196]. As DMD muscle continuously express chemokines, it is thought that muscle itself contributes to the chemotaxic process, causing the chronic inflammation; this way, the pathways activated by these molecules are considered interesting candidates for immunosuppressive therapies. Other treatments using new immunomodulatory drugs such as chemokine-receptor antibodies or cytokines neutralizing antibodies (Infliximab and Etanercept) are still confined to preclinical studies, although they seem promising in dystrophic animal models. In addition, it is known that MCSc regulate biological processes associated with inflammation and suppress various immune functions, through release of immune suppressive cytokines and production of soluble HLA-G [188]. As we discussed above M1/M2 balance influences the inflammatory environment; we previously demonstrated an amelioration of dystrophic features in scidA/AJ

mice consequent to T and B depletion and M2 macrophages switch [17]. Similarly, the group of Tidball demonstrated that anti-inflammatory IL-10 reduced the pathology of mdx muscular dystrophy by deactivating M1 macrophages [12, 197]. Conversely, an inflammatory environment could be beneficial in cell therapy as injected cells could be attracted to the site of injury and they could be facilitated in the chemotaxic process by chemokines/cytokines.

In conclusion, the ability to regulate the expression level of a therapeutic gene and to control the immune response is vital to proceed with gene therapy in clinic. Gene replacement strategies offer the potential for long-term correction. Improved gene therapy vectors together with advances in bypassing immune responses provide a platform for meaningful translation to patients. In the field of muscular dystrophies the combination of cell and gene therapy is the most promising as it is important to overcome the regenerative potential exhaustion. Engineered stem cells could provide new muscular fibers and decrease the immune response of the host; particularly, the application of myogenic stem cells as a possible cell or gene-cell combined therapy represents a very interesting tool, especially in tissues that are characterized by a chronic inflammation. However, we need to better clarify the immunopathogenetic mechanism underlying the different forms of MDs in order to develop more selective immunotherapies combined with cell and gene therapy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This work was supported by Associazione La Nostra Famiglia Fondo DMD Gli Amici di Emanuele, Associazione "Amici del Centro Dino Ferrari", EU's 7th Framework programme Optistem 223098, Ministry of Health (RF-2009-1547384), and Ystem s.r.l.

References

[1] A. E. H. Emery, "The muscular dystrophies," *British Medical Journal*, vol. 317, no. 7164, pp. 991–995, 1998.

[2] A. Bashir, Y.-T. Liu, B. J. Raphael, D. Carson, and V. Bafna, "Optimization of primer design for the detection of variable genomic lesions in cancer," *Bioinformatics*, vol. 23, no. 21, pp. 2807–2815, 2007.

[3] D. Gabellini, G. D'Antona, M. Moggio et al., "Facioscapulohumeral muscular dystrophy in mice overexpressing FRG1," *Nature*, vol. 439, no. 7079, pp. 973–977, 2006.

[4] S. Gordon and P. R. Taylor, "Monocyte and macrophage heterogeneity," *Nature Reviews Immunology*, vol. 5, no. 12, pp. 953–964, 2005.

[5] D. C. Guttridge, C. Albanese, J. Y. Reuther, R. G. Pestell, and A. S. Baldwin Jr., "NF-κB controls cell growth and differentiation through transcriptional regulation of cyclin D1," *Molecular and Cellular Biology*, vol. 19, no. 8, pp. 5785–5799, 1999.

[6] J. G. Tidball and S. A. Villalta, "Regulatory interactions between muscle and the immune system during muscle regeneration," *American Journal of Physiology: Regulatory Integrative and Comparative Physiology*, vol. 298, no. 5, pp. R1173–R1187, 2010.

[7] L. Arnold, A. Henry, F. Poron et al., "Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis," *Journal of Experimental Medicine*, vol. 204, no. 5, pp. 1057–1069, 2007.

[8] V. A. Fadok, D. L. Bratton, L. Guthrie, and P. M. Henson, "Differential effects of apoptotic versus lysed cells on macrophage production of cytokines: role of proteases," *Journal of Immunology*, vol. 166, no. 11, pp. 6847–6854, 2001.

[9] J. G. Tidball and M. Wehling-Henricks, "Macrophages promote muscle membrane repair and muscle fibre growth and regeneration during modified muscle loading in mice in vivo," *Journal of Physiology*, vol. 578, no. 1, pp. 327–336, 2007.

[10] S. Gordon, "Alternative activation of macrophages," *Nature Reviews Immunology*, vol. 3, no. 1, pp. 23–35, 2013.

[11] V. Horsley, K. M. Jansen, S. T. Mills, and G. K. Pavlath, "IL-4 acts as a myoblast recruitment factor during mammalian muscle growth," *Cell*, vol. 113, no. 4, pp. 483–494, 2003.

[12] S. A. Villalta, H. X. Nguyen, B. Deng, T. Gotoh, and J. G. Tidbal, "Shifts in macrophage phenotypes and macrophage competition for arginine metabolism affect the severity of muscle pathology in muscular dystrophy," *Human Molecular Genetics*, vol. 18, no. 3, pp. 482–496, 2009.

[13] R. M. McDouall, M. J. Dunn, and V. Dubowitz, "Nature of the mononuclear infiltrate and the mechanism of muscle damage in juvenile dermatomyositis and Duchenne muscular dystrophy," *Journal of the Neurological Sciences*, vol. 99, no. 2-3, pp. 199–217, 1990.

[14] E. Gussoni, G. K. Pavlath, R. G. Miller et al., "Specific T cell receptor gene rearrangements at the site of muscle degeneration in Duchenne muscular dystrophy," *Journal of Immunology*, vol. 153, no. 10, pp. 4798–4805, 1994.

[15] J. R. Mendell, K. Campbell, L. Rodino-Klapac et al., "Dystrophin immunity in Duchenne's muscular dystrophy," *The New England Journal of Medicine*, vol. 363, no. 15, pp. 1429–1437, 2010.

[16] A. Farini, M. Meregalli, M. Belicchi et al., "T and B lymphocyte depletion has a marked effect on the fibrosis of dystrophic skeletal muscles in the scid/mdx mouse," *Journal of Pathology*, vol. 213, no. 2, pp. 229–238, 2007.

[17] A. Farini, C. Sitzia, C. Navarro et al., "Absence of T and B lymphocytes modulates dystrophic features in dysferlin deficient animal model," *Experimental Cell Research*, vol. 318, no. 10, pp. 1160–1174, 2012.

[18] M. J. Spencer, E. Montecino-Rodriguez, K. Dorshkind, and J. G. Tidball, "Helper (CD4+) and cytotoxic (CD8+) T cells promote the pathology of dystrophin-deficient muscle," *Clinical Immunology*, vol. 98, no. 2, pp. 235–243, 2001.

[19] B. de Paepe and J. L. de Bleecker, "Cytokines and chemokines as regulators of skeletal muscle inflammation: presenting the case of Duchenne muscular dystrophy," *Mediators of Inflammation*, vol. 2013, Article ID 540370, 2013.

[20] P. Vandenabeele, L. Galluzzi, T. Vanden Berghe, and G. Kroemer, "Molecular mechanisms of necroptosis: an ordered cellular explosion," *Nature Reviews Molecular Cell Biology*, vol. 11, no. 10, pp. 700–714, 2010.

[21] D. Burzyn, W. Kuswanto, D. Kolodin et al., "A special population of regulatory T cells potentiates muscle repair," *Cell*, vol. 155, no. 6, pp. 1282–1295, 2013.

[22] K. Nagaraju, R. Rawat, E. Veszelovszky et al., "Dysferlin deficiency enhances monocyte phagocytosis: a model for the inflammatory onset of limb-girdle muscular dystrophy 2B," *American Journal of Pathology*, vol. 172, no. 3, pp. 774–785, 2008.

[23] R. Rawat, T. V. Cohen, B. Ampong et al., "Inflammasome up-regulation and activation in dysferlin-deficient skeletal muscle," *American Journal of Pathology*, vol. 176, no. 6, pp. 2891–2900, 2010.

[24] R. Han and K. P. Campbell, "Dysferlin and muscle membrane repair," *Current Opinion in Cell Biology*, vol. 19, no. 4, pp. 409–416, 2007.

[25] K. Nagaraju, N. Raben, G. Merritt, L. Loeffler, K. Kirk, and P. Plotz, "A variety of cytokines and immunologically relevant surface molecules are expressed by normal human skeletal muscle cells under proinflammatory stimuli," *Clinical and Experimental Immunology*, vol. 113, no. 3, pp. 407–414, 1998.

[26] A. M. Emslie-Smith, K. Arahata, and A. G. Engel, "Major histocompatibility complex class I antigen expression, immunolocalization of interferon subtypes, and T cell-mediated cytotoxicity in myopathies," *Human Pathology*, vol. 20, no. 3, pp. 224–231, 1989.

[27] N. Goebels, D. Michaelis, H. Wekerle, and R. Hohlfeld, "Human myoblasts as antigen-presenting cells," *Journal of Immunology*, vol. 149, no. 2, pp. 661–667, 1992.

[28] K. Arahata, T. Ishihara, H. Fukunaga et al., "Inflammatory response in facioscapulohumeral muscular dystrophy (FSHD): immunocytochemical and genetic analyses," *Muscle and Nerve*, vol. 18, no. 2, pp. S56–S66, 1995.

[29] T. L. Munsat, D. Piper, P. Cancilla, and J. Mednick, "Inflammatory myopathy with facioscapulohumeral distribution," *Neurology*, vol. 22, no. 4, pp. 335–347, 1972.

[30] R. Tawil, M. P. McDermott, S. Pandya et al., "A pilot trial of prednisone in facioscapulohumeral muscular dystrophy," *Neurology*, vol. 48, no. 1, pp. 46–49, 1997.

[31] S. Sookhoo, I. MacKinnon, K. Bushby, P. F. Chinnery, and D. Birchall, "MRI for the demonstration of subclinical muscle involvement in muscular dystrophy," *Clinical Radiology*, vol. 62, no. 2, pp. 160–165, 2007.

[32] G. Tasca, M. Pescatori, M. Monforte et al., "Different molecular signatures in magnetic resonance imaging-staged facioscapulohumeral muscular dystrophy muscles," *PLoS ONE*, vol. 7, no. 6, Article ID e38779, 2012.

[33] G. Frisullo, R. Frusciante, V. Nociti et al., "CD8+ T cells in facioscapulohumeral muscular dystrophy patients with inflammatory features at muscle MRI," *Journal of Clinical Immunology*, vol. 31, no. 2, pp. 155–166, 2011.

[34] A. Brunn, R. Schröder, and M. Deckert, "The inflammatory reaction pattern distinguishes primary dysferlinopathies from idiopathic inflammatory myopathies: an important role for the membrane attack complex," *Acta Neuropathologica*, vol. 112, no. 3, pp. 325–332, 2006.

[35] M. R. Hussein, S. A. Hamed, M. G. Mostafa, E. E. Abu-Dief, N. F. Kamel, and M. R. Kandil, "The effects of glucocorticoid therapy on the inflammatory and Dendritic cells in muscular dystrophies," *International Journal of Experimental Pathology*, vol. 87, no. 6, pp. 451–461, 2006.

[36] J. R. Mendell, L. Rodino-Klapac, Z. Sahenk et al., "Gene therapy for muscular dystrophy: lessons learned and path forward," *Neuroscience Letters*, vol. 527, no. 2, pp. 90–99, 2012.

[37] M. D. Grounds and J. Torrisi, "Anti-TNFα (Remicade) therapy protects dystrophic skeletal muscle from necrosis," *FASEB Journal*, vol. 18, no. 6, pp. 676–682, 2004.

[38] S. Hodgetts, H. Radley, M. Davies, and M. D. Grounds, "Reduced necrosis of dystrophic muscle by depletion of host neutrophils, or blocking TNFα function with Etanercept in mdx mice," *Neuromuscular Disorders*, vol. 16, no. 9-10, pp. 591–602, 2006.

[39] A. P. T. Taniguti, A. Pertille, C. Y. Matsumura, H. S. Neto, and M. J. Marques, "Prevention of muscle fibrosis and myonecrosis in mdxmice by suramin, a TGF-β1 blocker," *Muscle and Nerve*, vol. 43, no. 1, pp. 82–87, 2011.

[40] C. van Erp, N. G. Irwin, and A. J. Hoey, "Long-term administration of pirfenidone improves cardiac function in mdx mice," *Muscle and Nerve*, vol. 34, no. 3, pp. 327–334, 2006.

[41] E. P. Hoffman, D. Rao, and L. M. Pachman, "Clarifying the boundaries between the inflammatory and dystrophic myopathies: insights from molecular diagnostics and microarrays," *Rheumatic Disease Clinics of North America*, vol. 28, no. 4, pp. 743–757, 2002.

[42] M. C. Walter, P. Reilich, S. Thiele et al., "Treatment of dysferlinopathy with deflazacort: a double-blind, placebo-controlled clinical trial," *Orphanet Journal of Rare Diseases*, pp. 8–26, 2010.

[43] A. Lerario, F. Cogiamanian, C. Marchesi et al., "Effects of rituximab in two patients with dysferlin-deficient muscular dystrophy," *BMC Musculoskeletal Disorders*, vol. 11, article 157, 2010.

[44] H. Nemoto, S. Konno, H. Sugimoto et al., "Anti-TNF therapy using etanercept suppresses degenerative and inflammatory changes in skeletal muscle of older SJL/J mice," *Experimental and Molecular Pathology*, vol. 90, no. 3, pp. 264–270, 2011.

[45] O. Halevy, O. Genin, H. Barzilai-Tutsch et al., "Inhibition of muscle fibrosis and improvement of muscle histopathology in dysferlin knock-out mice treated with halofuginone," *Histology and Histopathology*, vol. 28, no. 2, pp. 211–2126, 2011.

[46] F. Mingozzi and K. A. High, "Immune responses to AAV vectors: overcoming barriers to successful gene therapy," *Blood*, vol. 122, no. 1, pp. 23–36, 2013.

[47] A. Asokan, D. V. Schaffer, and R. J. Samulski, "The AAV vector toolkit: poised at the clinical crossroads," *Molecular Therapy*, vol. 20, no. 4, pp. 699–708, 2012.

[48] Y. Yi, M. J. Noh, and K. H. Lee, "Current advances in retroviral gene therapy," *Current Gene Therapy*, vol. 11, no. 3, pp. 218–228, 2011.

[49] A.-K. Zaiss, Q. Liu, G. P. Bowen, N. C. W. Wong, J. S. Bartlett, and D. A. Muruve, "Differential activation of innate immune responses by adenovirus and adeno-associated virus vectors," *Journal of Virology*, vol. 76, no. 9, pp. 4580–4590, 2002.

[50] H. Li, N. Malani, S. R. Hamilton et al., "Assessing the potential for AAV vector genotoxicity in a murine model," *Blood*, vol. 117, no. 12, pp. 3311–3319, 2011.

[51] A. K. Zaiss and D. A. Muruve, "Immunity to adeno-associated virus vectors in animals and humans: a continued challenge," *Gene Therapy*, vol. 15, no. 11, pp. 808–816, 2008.

[52] B. Balakrishnan, D. Sen, S. Hareendran et al., "Activation of the cellular unfolded protein response by recombinant adeno-associated virus vectors," *PLoS ONE*, vol. 8, no. 1, Article ID e53845, 2013.

[53] A. T. Martino, M. Suzuki, D. M. Markusic et al., "The genome of self-complementary adeno-associated viral vectors increases Toll-like receptor 9-dependent innate immune responses in the liver," *Blood*, vol. 117, no. 24, pp. 6459–6468, 2011.

[54] S. Hareendran, B. Balakrishnan, D. Sen, S. Kumar, A. Srivastava, and G. R. Jayandharan, "Adeno-associated virus (AAV) vectors

in gene therapy: immune challenges and strategies to circumvent them," *Reviews in Medical Virology*, vol. 23, no. 6, pp. 399–413, 2013.

[55] R. Han, E. M. Frett, J. R. Levy et al., "Genetic ablation of complement C3 attenuates muscle pathology in dysferlin-deficient mice," *Journal of Clinical Investigation*, vol. 120, no. 12, pp. 4366–4374, 2010.

[56] A. K. Zaiss, M. J. Cotter, L. R. White et al., "Complement is an essential component of the immune response to adeno-associated virus vectors," *Journal of Virology*, vol. 82, no. 6, pp. 2727–2740, 2008.

[57] G. C. Pien, E. Basner-Tschakarjan, D. J. Hui et al., "Capsid antigen presentation flags human hepatocytes for destruction after transduction by adeno-associated viral vectors," *Journal of Clinical Investigation*, vol. 119, no. 6, pp. 1688–1695, 2009.

[58] C. Li, M. Hirsch, A. Asokan et al., "Adeno-associated virus type 2 (AAV2) capsid-specific cytotoxic T lymphocytes eliminate only vector-transduced cells coexpressing the AAV2 capsid in vivo," *Journal of Virology*, vol. 81, no. 14, pp. 7540–7547, 2007.

[59] C. Li, N. Diprimio, D. E. Bowles et al., "Single amino acid modification of adeno-associated virus capsid changes transduction and humoral immune profiles," *Journal of Virology*, vol. 86, no. 15, pp. 7752–7759, 2012.

[60] A. M. Maguire, F. Simonelli, E. A. Pierce et al., "Safety and efficacy of gene transfer for Leber's congenital amaurosis," *The New England Journal of Medicine*, vol. 358, no. 21, pp. 2240–2248, 2008.

[61] W. W. Hauswirth, T. S. Aleman, S. Kaushal et al., "Treatment of Leber congenital amaurosis due to RPE65 mutations by ocular subretinal injection of adeno-associated virus gene vector: short-term results of a phase I trial," *Human Gene Therapy*, vol. 19, no. 10, pp. 979–990, 2008.

[62] H. Jiang, D. Lillicrap, S. Patarroyo-White et al., "Multiyear therapeutic benefit of AAV serotypes 2, 6, and 8 delivering factor VIII to hemophilia A mice and dogs," *Blood*, vol. 108, no. 1, pp. 107–115, 2006.

[63] J. H. McIntosh, M. Cochrane, S. Cobbold et al., "Successful attenuation of humoral immunity to viral capsid and transgenic protein following AAV-mediated gene transfer with a non-depleting CD4 antibody and cyclosporine," *Gene Therapy*, vol. 19, no. 1, pp. 78–85, 2012.

[64] B. A. Valentine, B. J. Cooper, A. de Lahunta, R. O'Quinn, and J. T. Blue, "Canine X-linked muscular dystrophy. An animal model of Duchenne muscular dystrophy: clinical studies," *Journal of the Neurological Sciences*, vol. 88, no. 1–3, pp. 69–81, 1988.

[65] K. Yuasa, M. Yoshimura, N. Urasawa et al., "Injection of a recombinant AAV serotype 2 into canine skeletal muscles evokes strong immune responses against transgene products," *Gene Therapy*, vol. 14, no. 17, pp. 1249–1260, 2007.

[66] S. Adriouch, E. Franck, L. Drouot et al., "Improved immunological tolerance following combination therapy with CTLA-4/Ig and AAV-Mediated PD-L1/2 muscle gene transfer," *Frontiers in Microbiology*, vol. 2, p. 199, 2011.

[67] S. Lorain, D.-A. Gross, A. Goyenvalle, O. Danos, J. Davoust, and L. Garcia, "Transient immunomodulation allows repeated injections of AAV1 and correction of muscular dystrophy in multiple muscles," *Molecular Therapy*, vol. 16, no. 3, pp. 541–547, 2008.

[68] J. R. Mendell, L. R. Rodino-Klapac, X. Rosales-Quintero et al., "Limb-girdle muscular dystrophy type 2D gene therapy restores α-sarcoglycan and associated proteins," *Annals of Neurology*, vol. 66, no. 3, pp. 290–297, 2009.

[69] B. C. Schnepp, K. R. Clark, D. L. Klemanski, C. A. Pacak, and P. R. Johnson, "Genetic fate of recombinant adeno-associated virus vector genomes in muscle," *Journal of Virology*, vol. 77, no. 6, pp. 3495–3504, 2003.

[70] P. Gregorevic and J. S. Chamberlain, "Gene therapy for muscular dystrophy—a review of promising progress," *Expert Opinion on Biological Therapy*, vol. 3, no. 5, pp. 803–814, 2003.

[71] Z. Wang, T. Zhu, C. Qiao et al., "Adeno-associated virus serotype 8 efficiently delivers genes to muscle and heart," *Nature Biotechnology*, vol. 23, no. 3, pp. 321–328, 2005.

[72] A. Goyenvalle, A. Vulin, F. Fougerousse et al., "Rescue of dystrophic muscle through U7 snRNA-mediated exon skipping," *Science*, vol. 306, no. 5702, pp. 1796–1799, 2004.

[73] L. R. Rodino-Klapac, P. M. L. Janssen, C. L. Montgomery et al., "A translational approach for limb vascular delivery of the micro-dystrophin gene without high volume or high pressure for treatment of Duchenne muscular dystrophy," *Journal of Translational Medicine*, vol. 5, article 45, 2007.

[74] R. E. Bittner, S. Shorny, B. Streubel, C. Hubner, T. Voit, and W. Kress, "Serum antibodies to the deleted dystrophin sequence after cardiac transplantation in a patient with Becker's muscular dystrophy," *The New England Journal of Medicine*, vol. 333, no. 11, pp. 732–733, 1995.

[75] J. Huard, R. Roy, J.-P. Bouchard, F. Malouin, C. L. Richards, and J.-P. Tremblay, "Human myoblast transplantation between immunohistocompatible donors and recipients produces immune reactions," *Transplantation Proceedings*, vol. 24, no. 6, pp. 3049–3051, 1992.

[76] J. P. Tremblay, F. Malouin, R. Roy et al., "Results of a triple blind clinical study of myoblast transplantations without immunosuppressive treatment in young boys with Duchenne muscular dystrophy," *Cell Transplantation*, vol. 2, no. 2, pp. 99–112, 1993.

[77] A. Ferrer, K. E. Wells, and D. J. Wells, "Immune responses to dystrophin: implications for gene therapy of Duchenne muscular dystrophy," *Gene Therapy*, vol. 7, no. 17, pp. 1439–1446, 2000.

[78] E. Gussoni, H. M. Blau, and L. M. Kunkel, "The fate of individual myoblasts after transplantation into muscles of DMD patients," *Nature Medicine*, vol. 3, no. 97, p. 970, 1997.

[79] J. R. Mendell, J. T. Kissel, A. A. Amato et al., "Myoblast transfer in the treatment of Duchenne's muscular dystrophy," *The New England Journal of Medicine*, vol. 333, no. 13, pp. 832–838, 1995.

[80] K. Yuasa, M. Sakamoto, Y. Miyagoe-Suzuki et al., "Adeno-associated virus vector-mediated gene transfer into dystrophin-deficient skeletal muscles evokes enchanced immune response against the transgene product," *Gene Therapy*, vol. 9, no. 23, pp. 1576–1588, 2002.

[81] M. A. Khan, "Corticosteroid therapy in Duchenne muscular dystrophy," *Journal of the Neurological Sciences*, vol. 120, no. 1, pp. 8–14, 1993.

[82] K. M. Flanigan, K. Campbell, L. Viollet et al., "Antidystrophin T cell responses in Duchenne muscular dystrophy: prevalence and a glucocorticoid treatment effect," *Human Gene Therapy*, vol. 24, no. 9, pp. 797–806, 2013.

[83] N. A. Kootstra and I. M. Verma, "Gene Therapy with Viral Vectors," *Annual Review of Pharmacology and Toxicology*, vol. 43, pp. 413–439, 2003.

[84] T. Kafri, U. Blomer, D. A. Peterson, F. H. Gage, and I. M. Verma, "Sustained expression of genes delivered directly into liver and

muscle by lentiviral vectors," *Nature Genetics* , vol. 17, no. 3, pp. 314–317, 1997.

[85] D. A. Rubinson, C. P. Dillon, A. V. Kwiatkowski et al., "lentivirus-based system to functionally silence genes in primary mammalian cellsstem cells and transgenic mice by RNA interference," *Nature Genetics*, vol. 33, no. 3, pp. 401–406, 2003.

[86] R. G. Amado and I. S. Y. Chen, "Lentiviral vectors—the promise of gene therapy within reach?" *Science*, vol. 285, no. 5428, pp. 674–676, 1999.

[87] J. P. Greelish, L. T. Su, E. B. Lankford et al., "Stable restoration of the sarcoglycan complex in dystrophic muscle perfused with histamine and a recombinant adeno-associated viral vector," *Nature Medicine*, vol. 5, no. 4, pp. 439–443, 1999.

[88] J. Li, D. Dressman, Y. P. Tsao, A. Sakamoto, E. P. Hoffman, and X. Xiao, "rAAV vector-mediated sarcogylcan gene transfer in a hamster model for limb girdle muscular dystrophy," *Gene Therapy*, vol. 6, no. 1, pp. 74–82, 1999.

[89] A. A. Hack, C. T. Ly, F. Jiang et al., "γ-sarcoglycan deficiency leads to muscle membrane defects and apoptosis independent of dystrophin," *The Journal of Cell Biology*, vol. 142, no. 5, pp. 1279–1287, 1998.

[90] L. Cordier, A. A. Hack, M. O. Scott et al., "Rescue of skeletal muscles of γ-sarcoglycan-deficient mice with adeno-associated virus-mediated gene transfer," *Molecular Therapy*, vol. 1, no. 2, pp. 119–129, 2000.

[91] M. V. Neguembor and D. Gabellini, "In junk we trust: repetitive DNA, epigenetics and facioscapulohumeral muscular dystrophy," *Epigenomics*, vol. 2, no. 2, pp. 271–287, 2010.

[92] S. Bortolanza, A. Nonis, F. Sanvito et al., "AAV6-mediated systemic shRNA delivery reverses disease in a mouse model of facioscapulohumeral muscular dystrophy," *Molecular Therapy*, vol. 19, no. 11, pp. 2055–2064, 2011.

[93] R. L. Boudreau, I. Martins, and B. L. Davidson, "Artificial MicroRNAs as siRNA shuttles: improved safety as compared to shRNAs in vitro and in vivo," *Molecular Therapy*, vol. 17, no. 1, pp. 169–175, 2009.

[94] D. Grimm, K. L. Streetz, C. L. Jopling et al., "Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways," *Nature*, vol. 441, no. 7092, pp. 537–541, 2006.

[95] J. Meng, F. Muntoni, and J. E. Morgan, "Stem cells to treat muscular dystrophies—where are we?" *Neuromuscular Disorders*, vol. 21, no. 1, pp. 4–12, 2011.

[96] M. Amit and J. Itskovitz-Eldor, "Derivation and spontaneous differentiation of human embryonic stem cells," *Journal of Anatomy*, vol. 200, no. 3, pp. 225–232, 2002.

[97] M. K. Carpenter, E. Rosler, and M. S. Rao, "Characterization and differentiation of human embryonic stem cells," *Cloning and Stem Cells*, vol. 5, no. 1, pp. 79–88, 2003.

[98] L. M. Hoffman and M. K. Carpenter, "Characterization and culture of human embryonic stem cells," *Nature Biotechnology*, vol. 23, no. 6, pp. 699–708, 2005.

[99] S. Bhagavati and W. Xu, "Generation of skeletal muscle from transplanted embryonic stem cells in dystrophic mice," *Biochemical and Biophysical Research Communications*, vol. 333, no. 2, pp. 644–649, 2005.

[100] H. Kamochi, M. S. Kurokawa, H. Yoshikawa et al., "Transplantation of myocyte precursors derived from embryonic stem cells transfected with IGFII gene in a mouse model of muscle injury," *Transplantation*, vol. 82, no. 4, pp. 516–526, 2006.

[101] K. Takahashi and S. Yamanaka, "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors," *Cell*, vol. 126, no. 4, pp. 663–676, 2006.

[102] P. E. de Almeida, J. D. Ransohoff, A. Nahid, and J. C. Wu, "Immunogenicity of pluripotent stem cells and their derivatives," *Circulation Research*, vol. 112, no. 3, pp. 549–561, 2013.

[103] R.-J. Swijnenburg, S. Schrepfer, F. Cao et al., "In vivo imaging of embryonic stem cells reveals patterns of survival and immune rejection following transplantation," *Stem Cells and Development*, vol. 17, no. 6, pp. 1023–1029, 2008.

[104] T. Zhao, Z.-N. Zhang, Z. Rong, and Y. Xu, "Immunogenicity of induced pluripotent stem cells," *Nature*, vol. 474, no. 7350, pp. 212–215, 2011.

[105] A. Mullally and J. Ritz, "Beyond HLA: the significance of genomic variation for allogeneic hematopoietic stem cell transplantation," *Blood*, vol. 109, no. 4, pp. 1355–1362, 2007.

[106] Y. Mayshar, U. Ben-David, N. Lavon et al., "Identification and classification of chromosomal aberrations in human induced pluripotent stem cells," *Cell Stem Cell*, vol. 7, no. 4, pp. 521–531, 2010.

[107] F. Cao, S. Lin, X. Xie et al., "In vivo visualization of embryonic stem cell survival, proliferation, and migration after cardiac delivery," *Circulation*, vol. 113, no. 7, pp. 1005–1014, 2006.

[108] M. Drukker, G. Katz, A. Urbach et al., "Characterization of the expression of MHC proteins in human embryonic stem cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 15, pp. 9864–9869, 2002.

[109] E. Goulmy, A. Termijtelen, B. A. Bradley, and J. J. van Rood, "Alloimmunity to human H-Y," *The Lancet*, vol. 2, no. 7996, p. 1206, 1976.

[110] G. Opelz, "Non-HLA transplantation immunity revealed by lymphocytotoxic antibodies," *The Lancet*, vol. 365, no. 9470, pp. 1570–1576, 2005.

[111] K. M. Dhodapkar, D. Feldman, P. Matthews et al., "Natural immunity to pluripotency antigen OCT4 in humans," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 19, pp. 8718–8723, 2010.

[112] P. J. Fairchild, S. Cartland, K. F. Nolan, and H. Waldmann, "Embryonic stem cells and the challenge of transplantation tolerance," *Trends in Immunology*, vol. 25, no. 9, pp. 465–470, 2004.

[113] S. Firner, L. Onder, V. Nindl, and B. Ludewig, "Tight control—decision-making during T cell-vascular endothelial cell interaction," *Frontiers in Immunology*, vol. 3, p. 279, 2012.

[114] T. A. Partridge, J. E. Morgan, G. R. Coulton, E. P. Hoffman, and L. M. Kunkel, "Conversion of mdx myofibres from dystrophin-negative to -positive by injection of normal myoblasts," *Nature*, vol. 337, no. 6203, pp. 176–179, 1989.

[115] D. Skuk and J. P. Tremblay, "Complement deposition and cell death after myoblast transplantation," *Cell Transplantation*, vol. 7, no. 5, pp. 427–434, 1998.

[116] G. Benichou and V. H. Price, "T cell repertoire in mice with alopecia areata," *Journal of Investigative Dermatology Symposium Proceedings*, vol. 4, no. 3, pp. 224–225, 1999.

[117] H. Gudmundsdottir and L. A. Turka, "T cell costimulatory blockade: new therapies for transplant rejection," *Journal of the American Society of Nephrology*, vol. 10, no. 6, pp. 1356–1365, 1999.

[118] P. C. Nahirney, P. R. Dow, and W. K. Ovalle, "Quantitative morphology of mast cells in skeletal muscle of normal and genetically dystrophic mice," *The Anatomical Record*, vol. 247, no. 3, pp. 341–349, 1997.

[119] G. M. Smythe, S. I. Hodgetts, and M. D. Grounds, "Immunobiology and the future of myoblast transfer therapy," *Molecular Therapy*, vol. 1, no. 4, pp. 304–313, 2000.

[120] G. Karpati, D. Ajdukovic, D. Arnold et al., "Myoblast transfer in Duchenne muscular dystrophy," *Annals of Neurology*, vol. 34, no. 1, pp. 8–17, 1993.

[121] P. K. Law, T. G. Goodwin, Q. Fang et al., "Cell transplantation as an experimental treatment for Duchenne muscular dystrophy," *Cell Transplantation*, vol. 2, no. 6, pp. 485–505, 1993.

[122] R. G. Miller, K. R. Sharma, G. K. Pavlath et al., "Myoblast implantation in Duchenne muscular dystrophy: the San Francisco study," *Muscle Nerve*, vol. 20, no. 4, pp. 469–478, 1997.

[123] D. Skuk, M. Goulet, B. Roy et al., "First test of a "high-density injection" protocol for myogenic cell transplantation throughout large volumes of muscles in a Duchenne muscular dystrophy patient: eighteen months follow-up," *Neuromuscular Disorders*, vol. 17, no. 1, pp. 38–46, 2007.

[124] D. Charron, C. Suberbielle-Boissel, and R. Al-Daccak, "Immunogenicity and allogenicity: a challenge of stem cell therapy," *Journal of Cardiovascular Translational Research*, vol. 2, no. 1, pp. 130–138, 2009.

[125] M. Drukker and N. Benvenisty, "The immunogenicity of human embryonic stem-derived cells," *Trends in Biotechnology*, vol. 22, no. 3, pp. 136–141, 2004.

[126] D. Charron, C. Suberbielle-Boissel, R. Tamouza, and R. Al-Daccak, "Anti-HLA antibodies in regenerative medicine stem cell therapy," *Human Immunology*, vol. 73, no. 12, pp. 1287–1294, 2012.

[127] M. R. Clatworthy, M. Espeli, N. Torpey, and K. G. C. Smith, "The generation and maintenance of serum alloantibody," *Current Opinion in Immunology*, vol. 22, no. 5, pp. 669–681, 2010.

[128] F. Li, M. E. Atz, and E. F. Reed, "Human leukocyte antigen antibodies in chronic transplant vasculopathy-mechanisms and pathways," *Current Opinion in Immunology*, vol. 21, no. 5, pp. 557–562, 2009.

[129] R. Al-Daccak, N. Mooney, and D. Charron, "MHC class II signaling in antigen-presenting cells," *Current Opinion in Immunology*, vol. 16, no. 1, pp. 108–113, 2004.

[130] G. Barbieri, E. Rimini, and M. A. Costa, "Effects of human leukocyte antigen (HLA)-DR engagement on melanoma cells," *International Journal of Oncology*, vol. 38, no. 6, pp. 1589–1595, 2011.

[131] N. Bertho, V. M. Blancheteau, N. Setterblad et al., "MHC class II-mediated apoptosis of mature dendritic cells proceeds by activation of the protein kinase C-δ isoenzyme," *International Immunology*, vol. 14, no. 8, pp. 935–942, 2002.

[132] A. Asakura and M. A. Rudnicki, "Side population cells from diverse adult tissues are capable of in vitro hematopoietic differentiation," *Experimental Hematology*, vol. 30, no. 11, pp. 1339–1345, 2002.

[133] R. Benchaouir, M. Meregalli, A. Farini et al., "Restoration of human dystrophin following transplantation of exon-skipping-engineered DMD patient stem cells into dystrophic mice," *Cell Stem Cell*, vol. 1, no. 6, pp. 646–657, 2007.

[134] G. Cossu and P. Bianco, "Mesoangioblasts - Vascular progenitors for extravascular mesodermal tissues," *Current Opinion in Genetics and Development*, vol. 13, no. 5, pp. 537–542, 2003.

[135] M. Dezawa, H. Ishikawa, Y. Itokazu et al., "Developmental biology: bone marrow stromal cells generate muscle cells and repair muscle degeneration," *Science*, vol. 309, no. 5732, pp. 314–317, 2005.

[136] M. J. Doherty, B. A. Ashton, S. Walsh, J. N. Beresford, M. E. Grant, and A. E. Canfield, "Vascular pericytes express osteogenic potential in vitro and in vivo," *Journal of Bone and Mineral Research*, vol. 13, no. 5, pp. 828–838, 1998.

[137] M. Gavina, M. Belicchi, B. Rossi et al., "VCAM-1 expression on dystrophic muscle vessels has a critical role in the recruitment of human blood-derived CD133+ stem cells after intra-arterial transplantation," *Blood*, vol. 108, no. 8, pp. 2857–2866, 2006.

[138] Z. Qu-Petersen, B. Deasy, R. Jankowski et al., "Identification of a novel population of muscle stem cells in mice: potential for muscle regeneration," *The Journal of Cell Biology*, vol. 157, no. 5, pp. 851–864, 2002.

[139] B. G. Galvez, M. Sampaolesi, S. Brunelli et al., "Complete repair of dystrophic skeletal muscle by mesoangioblasts with enhanced migration ability," *The Journal of Cell Biology*, vol. 174, no. 2, pp. 231–243, 2006.

[140] Y. Torrente, G. Camirand, F. Pisati et al., "Identification of a putative pathway for the muscle homing of stem cells in a muscular dystrophy model," *The Journal of Cell Biology*, vol. 162, no. 3, pp. 511–520, 2003.

[141] A. J. Friedenstein, U. F. Gorskaja, and N. N. Kulagina, "Fibroblast precursors in normal and irradiated mouse hematopoietic organs," *Experimental Hematology*, vol. 4, no. 5, pp. 267–274, 1976.

[142] C. De Bari, F. Dell'Accio, P. Tylzanowski, and F. P. Luyten, "Multipotent mesenchymal stem cells from adult human synovial membrane," *Arthritis & Rheumatology*, vol. 44, no. 8, pp. 1928–1942, 2001.

[143] M. J. Hoogduijn, M. J. Crop, A. M. A. Peeters et al., "Human heart, spleen, and perirenal fat-derived mesenchymal stem cells have immunomodulatory capacities," *Stem Cells and Development*, vol. 16, no. 4, pp. 597–604, 2007.

[144] D. G. Phinney and D. J. Prockop, "Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair—current views," *Stem Cells*, vol. 25, no. 11, pp. 2896–2902, 2007.

[145] K.-G. Shyu, B.-W. Wang, H.-F. Hung, C.-C. Chang, and D. T.-B. Shih, "Mesenchymal stem cells are superior to angiogenic growth factor genes for improving myocardial performance in the mouse model of acute myocardial infarction," *Journal of Biomedical Science*, vol. 13, no. 1, pp. 47–58, 2006.

[146] S.-C. Hung, R. R. Pochampally, S.-C. Chen, S.-C. Hsu, and D. J. Prockop, "Angiogenic effects of human multipotent stromal cell conditioned medium activate the PI3K-Akt pathway in hypoxic endothelial cells to inhibit apoptosis, increase survival, and stimulate angiogenesis," *Stem Cells*, vol. 25, no. 9, pp. 2363–2370, 2007.

[147] K. English, F. P. Barry, and B. P. Mahon, "Murine mesenchymal stem cells suppress dendritic cell migration, maturation and antigen presentation," *Immunology Letters*, vol. 115, no. 1, pp. 50–58, 2008.

[148] A. J. Nauta, A. B. Kruisselbrink, E. Lurvink, R. Willemze, and W. E. Fibbe, "Mesenchymal stem cells inhibit generation and function of both CD34 +-derived and monocyte-derived dendritic cells," *Journal of Immunology*, vol. 177, no. 4, pp. 2080–2087, 2006.

[149] S. Beyth, Z. Borovsky, D. Mevorach et al., "Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T-cell unresponsiveness," *Blood*, vol. 105, no. 5, pp. 2214–2219, 2005.

[150] P. A. Sotiropoulou, S. A. Perez, A. D. Gritzapis, C. N. Baxevanis, and M. Papamichail, "Interactions between human mesenchymal stem cells and natural killer cells," *Stem Cells*, vol. 24, no. 1, pp. 74–85, 2006.

[151] G. M. Spaggiari, A. Capobianco, H. Abdelrazik, F. Becchetti, M. C. Mingari, and L. Moretta, "Mesenchymal stem

cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin E2," *Blood*, vol. 111, no. 3, pp. 1327–1333, 2008.

[152] G. M. Spaggiari, A. Capobianco, S. Becchetti, M. C. Mingari, and L. Moretta, "Mesenchymal stem cell-natural killer cell interactions: evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation," *Blood*, vol. 107, no. 4, pp. 1484–1490, 2006.

[153] P.-D. Johann, M. Vaegler, F. Gieseke et al., "Tumour stromal cells derived from paediatric malignancies display MSC-like properties and impair NK cell cytotoxicity," *BMC Cancer*, vol. 10, article 501, 2010.

[154] S. Asari, S. Itakura, K. Ferreri et al., "Mesenchymal stem cells suppress B-cell terminal differentiation," *Experimental Hematology*, vol. 37, no. 5, pp. 604–615, 2009.

[155] A. Augello, R. Tasso, S. M. Negrini et al., "Bone marrow mesenchymal progenitor cells inhibit lymphocyte proliferation by activation of the programmed death 1 pathway," *European Journal of Immunology*, vol. 35, no. 5, pp. 1482–1490, 2005.

[156] P. Comoli, F. Ginevri, R. MacCario et al., "Human mesenchymal stem cells inhibit antibody production induced in vitro by allostimulation," *Nephrology Dialysis Transplantation*, vol. 23, no. 4, pp. 1196–1202, 2008.

[157] A. Corcione, F. Benvenuto, E. Ferretti et al., "Human mesenchymal stem cells modulate B-cell functions," *Blood*, vol. 107, no. 1, pp. 367–372, 2006.

[158] K. Le Blanc, I. Rasmusson, C. Götherström et al., "Mesenchymal stem cells inhibit the expression of CD25 (interleukin-2 receptor) and CD38 on phytohaemagglutinin-activated lymphocytes," *Scandinavian Journal of Immunology*, vol. 60, no. 3, pp. 307–315, 2004.

[159] K. Sato, K. Ozaki, I. Oh et al., "Nitric oxide plays a critical role in suppression of T-cell proliferation by mesenchymal stem cells," *Blood*, vol. 109, no. 1, pp. 228–234, 2007.

[160] G. Ren, J. Su, L. Zhang et al., "Species variation in the mechanisms of mesenchymal stem cell-mediated immunosuppression," *Stem Cells*, vol. 27, no. 8, pp. 1954–1962, 2009.

[161] G. Ren, L. Zhang, X. Zhao et al., "Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide," *Cell Stem Cell*, vol. 2, no. 2, pp. 141–150, 2008.

[162] F. Casiraghi, N. Azzollini, P. Cassis et al., "Pretransplant infusion of mesenchymal stem cells prolongs the survival of a semiallogeneic heart transplant through the generation of regulatory T cells," *Journal of Immunology*, vol. 181, no. 6, pp. 3933–3946, 2008.

[163] J. Chan, S. N. Waddington, K. O'Donoghue et al., "Widespread distribution and muscle differentiation of human fetal mesenchymal stem cells after intrauterine transplantation in dystrophic mdx mouse," *Stem Cells*, vol. 25, no. 4, pp. 875–884, 2007.

[164] S.-W. Feng, X.-L. Lu, Z.-S. Liu et al., "Dynamic distribution of bone marrow-derived mesenchymal stromal cells and change of pathology after infusing into mdx mice," *Cytotherapy*, vol. 10, no. 3, pp. 254–264, 2008.

[165] T. C. MacKenzie, A. F. Shaaban, A. Radu, and A. W. Flake, "Engraftment of bone marrow and fetal liver cells after in utero transplantation in MDX mice," *Journal of Pediatric Surgery*, vol. 37, no. 7, pp. 1058–1064, 2002.

[166] E. J. Gang, R. Darabi, D. Bosnakovski et al., "Engraftment of mesenchymal stem cells into dystrophin-deficient mice is not accompanied by functional recovery," *Experimental Cell Research*, vol. 315, no. 15, pp. 2624–2636, 2009.

[167] A. Shabbir, D. Zisa, M. Leiker, C. Johnston, H. Lin, and T. Lee, "Muscular dystrophy therapy by nonautologous mesenchymal stem cells: muscle regeneration without immunosuppression and inflammation," *Transplantation*, vol. 87, no. 9, pp. 1275–1282, 2009.

[168] N. M. Vieira, M. Valadares, E. Zucconi et al., "Human adipose-derived mesenchymal stromal cells injected systemically into GRMD dogs without immunosuppression are able to reach the host muscle and express human dystrophin," *Cell Transplantation*, vol. 21, no. 7, pp. 1407–1417, 2012.

[169] F. Mingozzi, J. J. Meulenberg, D. J. Hui et al., "AAV-1-mediated gene transfer to skeletal muscle in humans results in dose-dependent activation of capsid-specific T cells," *Blood*, vol. 114, no. 10, pp. 2077–2086, 2009.

[170] A. C. Nathwani, E. G. D. Tuddenham, S. Rangarajan et al., "Adenovirus-associated virus vector-mediated gene transfer in hemophilia B," *The New England Journal of Medicine*, vol. 365, no. 25, pp. 2357–2365, 2011.

[171] E. Breous, S. Somanathan, and J. M. Wilson, "BALB/c mice show impaired hepatic tolerogenic response following AAV gene transfer to the liver," *Molecular Therapy*, vol. 18, no. 4, pp. 766–774, 2010.

[172] L. E. Mays, L. H. Vandenberghe, R. Xiao et al., "Adeno-associated virus capsid structure drives CD4-dependent CD8 + T cell response to vector encoded proteins," *Journal of Immunology*, vol. 182, no. 10, pp. 6051–6060, 2009.

[173] G. Gao, L. H. Vandenberghe, M. R. Alvira et al., "Clades of adeno-associated viruses are widely disseminated in human tissues," *Journal of Virology*, vol. 78, no. 12, pp. 6381–6388, 2004.

[174] G.-P. Gao, M. R. Alvira, L. Wang, R. Calcedo, J. Johnston, and J. M. Wilson, "Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 18, pp. 11854–11859, 2002.

[175] A. M. Andrés, M. J. Hubisz, A. Indap et al., "Targets of balancing selection in the human genome," *Molecular Biology and Evolution*, vol. 26, no. 12, pp. 2755–2764, 2009.

[176] K. Tang, K. R. Thornton, and M. Stoneking, "A new approach for using genome scans to detect recent positive selection in the human genome," *PLoS Biology*, vol. 5, no. 7, p. e171, 2007.

[177] D. Forni, R. Cagliani, C. Tresoldi et al., "An evolutionary analysis of antigen processing and presentation across different timescales reveals pervasive selection," *PLOS Genetics*, vol. 10, no. 3, Article ID e1004189, 2014.

[178] S. R. Grossman, I. Shylakhter, E. K. Karlsson et al., "A composite of multiple signals distinguishes causal variants in regions of positive selection," *Science*, vol. 327, no. 5967, pp. 883–886, 2010.

[179] B. F. Voight, S. Kudaravalli, X. Wen, and J. K. Pritchard, "A map of recent positive selection in the human genome," *PLoS Biology*, vol. 4, no. 3, p. e72, 2006.

[180] E. Schuller and A. Govaerts, "First results of immunotherapy with immunoglobulin G in multiple sclerosis patients," *European Neurology*, vol. 22, no. 3, pp. 205–212, 1983.

[181] P. Gajdos, H. Outin, D. Elkharrat et al., "High-dose intravenous gammaglobulin for myasthenia gravis," *The Lancet*, vol. 1, no. 8373, pp. 406–407, 1984.

[182] P. D. Donofrio, A. Berger, T. H. Brannagan III et al., "Consensus statement: the use of intravenous immunoglobulin in the treatment of neuromuscular conditions report of the AANEM Ad Hoc Committee," *Muscle and Nerve*, vol. 40, no. 5, pp. 890–900, 2009.

[183] A. Quick and R. Tandan, "Mechanisms of action of intravenous immunoglobulin in inflammatory muscle disease," *Current Rheumatology Reports*, vol. 13, no. 3, pp. 192–198, 2011.

[184] M. Dall'Era and J. Davis, "CTLA4Ig: a novel inhibitor of costimulation," *Lupus*, vol. 13, no. 5, pp. 372–376, 2004.

[185] P. Blaha, S. Bigenzahn, Z. Koporc, M. Sykes, F. Muehlbacher, and T. Wekerle, "Short-term immunosuppression facilitates induction of mixed chimerism and tolerance after bone marrow transplantation without cytoreductive conditioning," *Transplantation*, vol. 80, no. 2, pp. 237–243, 2005.

[186] R. Calcedo and J. M. Wilson, "Humoral immune response to AAV," *Frontiers in Immunology*, vol. 4, p. 341, 2013.

[187] Z. Wang, S. J. Tapscott, J. S. Chamberlain, and R. Storb, "Immunity and AAV-mediated gene therapy for muscular dystrophies in large animal models and human trials," *Frontiers in Immunology*, vol. 2, p. 201, 2011.

[188] T. E. Ichim, D. T. Alexandrescu, F. Solano et al., "Mesenchymal stem cells as anti-inflammatories: implications for treatment of Duchenne muscular dystrophy," *Cellular Immunology*, vol. 260, no. 2, pp. 75–82, 2010.

[189] Y. Torrente, M. Belicchi, C. Marchesi et al., "Autologous transplantation of muscle-derived CD133+ stem cells in Duchenne muscle patients," *Cell Transplantation*, vol. 16, no. 6, pp. 563–577, 2007.

[190] B. M. Deasy, B. M. Gharaibeh, J. B. Pollett et al., "Long-term self-renewal of postnatal muscle-derived stem cells," *Molecular Biology of the Cell*, vol. 16, no. 7, pp. 3323–3333, 2005.

[191] B. M. Deasy, R. J. Jankowski, and J. Huard, "Muscle-derived stem cells: characterization and potential for cell-mediated therapy," *Blood Cells, Molecules, and Diseases*, vol. 27, no. 5, pp. 924–933, 2001.

[192] M. Sampaolesi, S. Blot, G. D'Antona et al., "Mesoangioblast stem cells ameliorate muscle function in dystrophic dogs," *Nature*, vol. 444, no. 7119, pp. 574–579, 2006.

[193] M. Sampaolesi, Y. Torrente, A. Innocenzi et al., "Cell therapy of α-sarcoglycan null dystrophic mice through intra-arterial delivery of mesoangioblasts," *Science*, vol. 301, no. 5632, pp. 487–492, 2003.

[194] F. S. Tedesco, H. Hoshiya, D. 'Antona G et al., "Stem cell-mediated transfer of a human artificial chromosome ameliorates muscular dystrophy," *Science Translational Medicine*, vol. 3, no. 96, p. 96ra78, 2011.

[195] C. Figueiredo, D. Wedekind, T. Muller et al., "MHC universal cells survive in an allogeneic environment after incompatible transplantation," *BioMed Research International*, vol. 2013, Article ID 796046, 12 pages, 2013.

[196] A. Mariano, A. Henning, and R. Han, "Dysferlin-deficient muscular dystrophy and innate immune activation," *FEBS Journal*, vol. 280, no. 17, pp. 4165–4176, 2013.

[197] S. A. Villalta, C. Rinaldi, B. Deng, G. Liu, B. Fedor, and J. G. Tidball, "Interleukin-10 reduces the pathology of mdx muscular dystrophy by deactivating M1 macrophages and modulating macrophage phenotype," *Human Molecular Genetics*, vol. 20, no. 4, pp. 790–805, 2011.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 949730, 10 pages
http://dx.doi.org/10.1155/2014/949730

Review Article

Vitamin D Receptor Agonists: Suitable Candidates as Novel Therapeutic Options in Autoimmune Inflammatory Myopathy

Clara Crescioli

Section of Health Sciences, Department of Movement, Human and Health Sciences, University of Rome Foro Italico, 00135 Rome, Italy

Correspondence should be addressed to Clara Crescioli; clara.crescioli@uniroma4.it

Received 27 December 2013; Accepted 9 April 2014; Published 7 May 2014

Academic Editor: Marina Bouché

The primary aim in the treatment of autoimmune inflammatory myopathies (IMs) is to recover muscle function. The presence of immune/inflammatory cell infiltrates within muscle tissues represents the common feature of different IM subtypes, albeit a correlation between muscular damage extent and inflammation degree is often lacking. Treatments for IMs are based on life-long immunosuppressive therapy, with the well known adverse effects; recovery is incomplete for many patients. More effective therapies, with reduced side-effects, are highly desirable. Vitamin D receptor (VDR) agonists emerge to retain pleiotropic anti-inflammatory properties, since they regulate innate and adaptive immunity by switching the immune response from proinflammatory T helper 1 (Th1) type to tolerogenic T helper 2 (Th2) type dominance. In skeletal muscle cells less hypercalcemic VDR ligands target powerful mediators of inflammation, such as TNFα and TNFα driven paths, without affecting immune or muscle cells viability, retaining the potentiality to counteract Th1 driven overreactivity established by the self-enhancing inflammatory loop between immune and skeletal muscle cells. This review summarizes those features of VDR agonists as candidates in future treatment of IM.

1. Introduction

Increasing evidence points out that vitamin D, beside bone metabolism and calcium homeostasis regulation, plays a pivotal role in maintaining the functionality of many other tissues, including skeletal muscle. A direct association of vitamin D status with skeletal muscle fiber composition, muscle power and force, or physical performance has been documented by several studies in old or young human population [1, 2]; remarkably, vitamin D supplementation is associated with improvements in muscle performance and fall reduction [1, 3–6].

Experimental models of VDR null mutant mice document diffused muscle fiber abnormalities and severe alterations in muscle cell differentiation or fiber development/maturation [7–9]; in humans, VDR gene polymorphisms have been associated with muscle strength defects, as recently reported [1, 10]. Direct effects of vitamin D on muscle cell proliferation, differentiation, and myotube size have been recently proposed in a murine experimental *in vitro* model [11]. Skeletal muscle is a well known target tissue of vitamin D action and the association between severe vitamin D deficiency and myopathy has been recognized since and recently confirmed [1, 12]. Myopathy is characterized by severe myofiber degeneration and muscle wasting; in particular, IMs are a wide range of autoimmune diseases, collectively known as myositis, characterized clinically by reduced muscle endurance and weakness, chronic inflammation, and infiltration by immune/inflammatory cells in skeletal muscles. Since both adaptive and innate immunity are involved in IMs, the mainstay treatment is directed to suppress or modify immune cell activity and is based on high dose corticosteroid combined with immunosuppressive drugs, as steroid-sparing agents [13–15]. However, most of IM patients have just a partial clinical improvement, few recover muscle performance, and about 25% are refractory to those drugs and left with disability [13–15], suggesting that pharmacological targeting the immune system may be not enough for satisfactory therapeutic effects.

Much more interest has been recently addressed to the muscular component, as an active counterpart dialoguing with the immune system during inflammation throughout

the production of cytokines and chemokines, highly chemotactic peptides. In this light, skeletal muscle cells, behaving as immunoactive structures, could be also hypothesized to be therapeutic targets as well.

Advances in clinical and bench research highlight the vitamin D impact on muscle function and morphology, either in physiologic or pathologic conditions [16–18]; also, VDR agonists emerge to exert pleiotropic activities in (auto)immune regulation by targeting both immune and resident cells [19–21].

This review aims to offer an overview on VDR agonists as potential novel therapeutic tools to control inflammation in IMs; in particular, biomolecular pathway(s) and inflammatory mediators within skeletal muscle cells engaged in IM pathogenesis, such as the cytokine TNFα and the chemokine CXCL10, will be discussed as intracellular pharmacological target(s) of nonhypercalcemic VDR agonists.

2. Pathogenic Mechanisms of IMs

IMs are a heterogeneous group of systemic autoimmune diseases subclassified in distinct subgroups, that is, idiopathic dermatomyositis (DM), polymyositis (PM), inclusion body myositis (IBM), the most studied ones, necrotizing autoimmune myositis, and myositis associated with systemic disorders on the basis of some clinical and histological differences [22–24]. Muscle weakness, fatigue, and elevated serum muscle enzymes, together with myofiber degeneration/fibrosis and mononuclear cell infiltration represent, respectively, clinical and histological features common to all subtypes.

Different pathogenic mechanisms have been hypothesized due to distinct predominating localization/phenotype of the inflammatory infiltrates, that is, while a striking dominance of $CD4^+$ T cells has been reported at perivascular/perimysial sites, as often found in DM, endomysial infiltrates are dominated by $CD8^+$ T cells, as more frequently observed in PM and IBM; the presence of B lymphocytes, which seem to preferentially target the microvascular component in DM, is considered less critical in PM [25]. Those differences, however, appear to be an oversimplification of the reality: an overlap between clinical phenotypes, immunotypes, and histopathology has been often depicted and frequently mirrors an overlap in diagnostic criteria as well [15, 25–27]. The inflammatory molecules and mediators involved in muscles affected by myositis are highly similar, given that some essential molecular paths engaged in immune response (innate and adaptive) are shared between the different IM subsets. The presence of autoantibodies, frequently detected in PM and DM, autoreactive lymphocytes, together with overexpression of major histocompatibility complex (MHC, or HLA, human leucocyte antigen) molecules on the surface of the affected myofibers albeit, at different degree, represent common traits of immune-mediated diseases. HLA molecules mediate the immune response by presenting processed antigen peptides, either self- or not-self, to activated T cells. In particular, T cells with Th1 immune reaction predominance, macrophages, and dendritic cells (DC) are found in muscles of the different subgroups of IMs [25, 28]. The presence of B lymphocytes, natural killer (NK) cells, or antigen presenting cell (APC) other than DC—such as endothelial or skeletal muscle cells themselves—have been also observed in all types of IMs [29–31]. However, albeit pathogenic mechanisms still are unclear, Th1 cells and macrophages play a pivotal role in IM pathogenesis: their local accumulation likely contributes to the deposition of immune complexes within skeletal muscles [32, 33] through the release of functional molecules, such as cytokines and chemokines; once present within the muscular microenvironment, they are able to cause damages directly to fibers and capillaries of the affected muscles [34]. The expression of cytokines as IFNγ, TNFα, and several interleukins (IL), such as IL-6, IL-4, IL-17, or IL-12p40, is increased in muscle biopsies of IM subjects [35–38]. Interactions between cytokines/chemokines and lymphocytes, suggested to be the link between innate and adaptive immunity, are likely critical for IM type and stage [34]; in addition intrinsic mechanisms in skeletal muscle appear to be highly significant in IM pathogenesis as well.

3. Skeletal Muscle as an Immunoactive Organ

The immunocompetence of nonimmune tissues has been recognized as determinant to drive the course of immune-inflammatory processes in several diseases, from organ rejection to autoimmune pathologies [39, 40]. Skeletal muscle cells are nowadays considered not only targets of immunological injury but actual active structures with intrinsic immunological capabilities [41–43].

3.1. Chemokines and Cytokines in Skeletal Muscle. Skeletal muscle is now considered as a secretory organ able to produce and release some cytokines—also termed myokines within the specific tissue context—to communicate with other organs, either in physiological conditions, that is, under contraction [42], or pathological conditions, as in inflammatory processes [43].

In particular, chemokines, a class of small cytokines with potent chemotactic activity, such as IL-8 (CXCL8), Mig (CXCL9), IP-10 (CXCL10), RANTES (CCL5), and MCP-1 (CCL2), are overexpressed during myositis in infiltrating inflammatory cells, extracellular matrix, and muscle fibers [32, 44–47]. Those molecules seem to have relevance for the immune pathogenesis of IMs because they promote and facilitate activated Th1 type cell trafficking to muscle tissues.

In line with this view, we have previously confirmed the importance of TNFα in IMs, suggesting new molecular insight(s) involving the important role of the chemokine CXCL10 in muscular inflammation [48, 49].

3.2. CXCL10 in Skeletal Muscle Inflammation. CXCL10 (or IP-10 10 kD IFNγ-induced protein) is a small peptide of CXC chemokine subfamily known to modulate innate and adaptive immune responses by controlling leukocyte trafficking [40, 50]. It is secreted by several types of immune and resident cells under proinflammatory conditions [40]. It is known to polarize T cells towards Th1 type dominance and seems to be directly associated to disease pathogenesis: through local tissue accumulation, it triggers and perpetuates a self-promoting inflammatory loop by interacting with its

specific receptor CXCR3 on T, NK, B cells, macrophages, and DCs [40, 51–54]. Notably, human skeletal muscle cells challenged by inflammatory stimuli secrete significant amount of CXCL10 (virtually absent in basal condition), likely throughout a TNFα-driven mechanism: TNFα/TNFα receptor (TNFαR) system seems the critical one in promoting muscular inflammation at cellular level in human skeletal muscle cells, involving nuclear transcription factor κB (NF-κB), C-Jun NH2-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK) intracellular path activation [48, 49]. In particular, the specific blockage of NF-κB and JNK signaling significantly reduced CXCL10 secretion [49]. Thus, albeit CXCL10 is by definition an IFNγ-induced chemokine, it seems to be driven almost exclusively by TNFα in inflammatory processes within human skeletal muscle cells. That is, in our opinion, not so surprising.

3.3. The Route of TNFα. TNFα is known as one of the essential cytokines in promoting muscular inflammation at cellular level [55, 56]; it has been recently confirmed to be a key mediator involved in IM pathogenesis and, consequently, emerges as a potential therapeutic target [57–59]. Accordingly, the neutralization of TNFα activity with specific antibodies, TNFαR antagonists, or NF-κB inhibitors has been investigated in experimental animal models of myositis, *in vitro* and *in vivo* [60, 61]. Of notice, TNFα pharmacological blockade by the neutralizing antibody infliximab or the soluble TNFαR etanercept, already used in clinics for the treatment of rheumatoid arthritis or Crohn's disease, has been extended to IMs, although with some caution [62–65]. Thus far, as from our and other's studies, targeting TNFα, TNFαR II—the subtype mainly engaged in immune response regulation [48, 66, 67]—and TNFα-related pathways—directly associated with CXCL10 production by skeletal muscle cells [49]—might be beneficial for IM treatment.

Hence, the capability of some VDR agonists to target TNFα, as shown by different studies, seems quite intriguing, and we would like to point out VDR agonist skill to selectively impair TNFα signaling during inflammation processes in human skeletal muscle cells.

4. VDR Agonists as Protolerogenic Molecules

It is known that vitamin D plays a role in the control of immune cell function through VDR, with important effects onto the immune-mediated response toward protolerogenic dominance [68–72]. VDR agonists are able to attenuate excessive Th1-driven inflammation and avoid downstream Th1 polarization during inflammatory processes involved in allo- or autoimmune response, that is, in organ transplant rejection or autoimmune diseases, as recently addressed in a review on the topic [39].

Herein, we would like to underline that the protolerogenic activity of VDR relies on their capability to control maturation, differentiation, and activation of different type of immune cells, that is, monocytes, macrophages, B and T lymphocytes, neutrophils, and DC, throughout the activation of VDR, either constitutively present or induced in the majority of the immune cells [73, 74].

Since the pioneering studies by Bhalla et al. [75], VDR agonists have been documented to inhibit selectively Th1 cell development [76, 77] and directly Th1-type cytokines, such as IL-2 and IFNγ [78–80]. In particular, while IL-2 inhibition is linked to an impairment of the transcription factor nuclear factor of activated T cells (NF-AT) complex formation, VDR ligand-induced IFNγ negative regulation has been explained by a direct interaction of the ligand-bound VDR complex with vitamin D responsive element (VDRE) within IFNγ promoter [78–80]. However, some controversy arises against direct effects of VDR ligands onto IFNγ inhibition [81]. Furthermore, T-cell activity can be inhibited by a VDR-mediated indirect mechanism through the downregulation of the expression of the MHC class II molecules and CD40, CD80, and CD86 costimulatory proteins in DC. DC are, indeed, well known targets of VDR ligands, which markedly impair IL-12—by targeting NF-κB, through Rel-B and c-Rel NFκB-related protein [82, 83]—and increase IL-10 production [81, 84–86]. The prevention of DC differentiation and maturation, activation, and survival leads to DC protolerogenic phenotype and function along with T-cell hyporesponsiveness, as shown by *in vivo* and *in vitro* studies [87, 88]. By the induction of protolerogenic DC phenotype, VDR ligands seem responsible for $CD4^+CD25^+$ regulatory T-cell enhancement [89, 90].

In addition, it is quite clear that VDR ligands directly target also Th17 cell subtype, as shown by the reduction of Th17 cytokines, such as IL-17A, IL-17F, and IL-22 by memory T cells in patients with early rheumatoid arthritis (RA) [91].

An enhanced development of Th2 type cells by VDR agonists throughout a direct effect on naïve $CD4^+$ cells has been reported [92]. A direct enhancement of Th2 type genes (i.e., IL-10, IL-4) is favored by VDR agonists while gene transcription of Th1/Th17 type cytokines (i.e., IL-2, IL-6, IL-12, IL-17, and IL-23) is declined [93, 94]. Therefore, a definite switch of Th1 cell response towards Th2-mediated events occurs. Moreover, since macrophages [91, 95, 96], DC [97], and T cells [91] produce $1,25(OH)_2D_3$, a contribution of this hormone to physiologically regulate innate and adaptive immunity could be speculated.

B cells, due to CYP27b1 (1alpha-hydroxylase) expression, have been hypothesized to be capable of autocrine/intracrine synthesis/response to vitamin D as well [98, 99]. In particular, vitamin D seems to predominantly modulate human naïve B cells activation through the VDR target gene cyp24a1 and NF-κB [99] regulation.

So far, the anti-inflammatory feature of VDR agonists exerted onto several types of immune cells depends not only on VDR expression, but, and maybe especially, on the presence of common targets in their signal transduction pathways, such as the NF-κB, downstream of TNFα [83, 100]. NF-κB is a key mediator of cytokine/chemokine-induced inflammation in several types of tissue resident cells and, notably a VDR agonist tissue target as well [48, 101–103] (Figure 1).

5. VDR Agonists as Therapeutic Tools in IMs

Based on the capacity to counteract NF-kB activation also in resident cells, VDR agonists cause a significant reduction

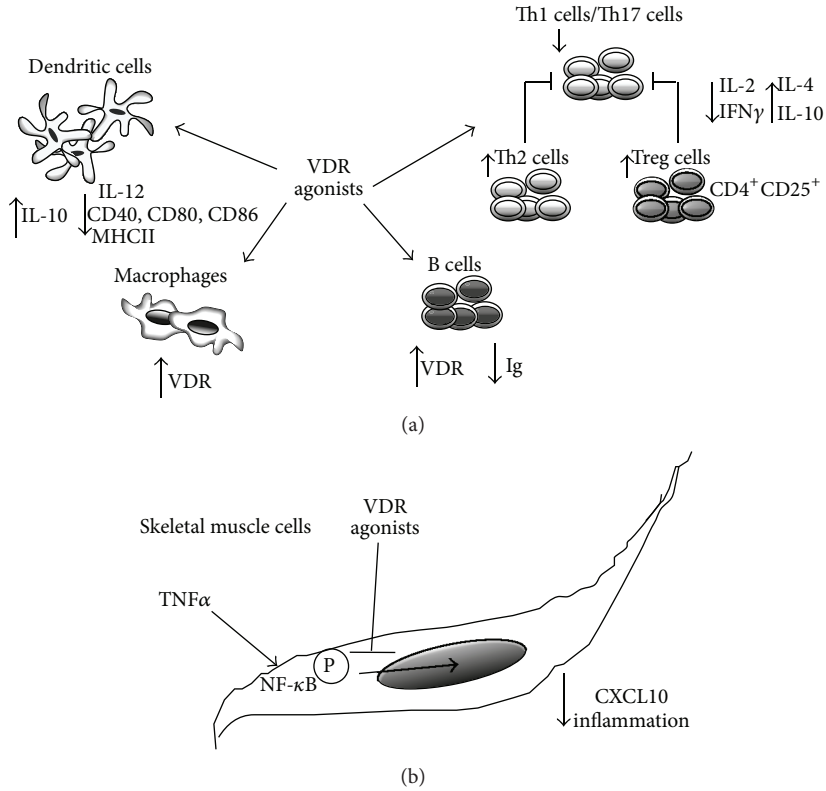


Figure 1: Schematic representation of protolerogenic effects of VDR ligands onto (a) immune cells, such as Th1/Th17, Th2, Treg subsets, DC, and macrophages and (b) skeletal muscle cells. Common targets, such as NF-κB, are involved to attenuate inflammation and promote a shift towards Th2 protolerogenic subtype dominance.

in local release of potent chemotactic factors—cytokines and chemokines—that, in turn, reduces the recruitment of immune cells (Th1 cells, macrophages, and DC) to the site of inflammation [18, 19, 80, 83]. As a result, the mechanisms underlying the self-enhancing inflammatory loop between immune and resident cells are likely impaired. So far, the feature of VDR agonists to counteract the path downstream of TNFα appears particularly relevant in view of a break up during inflammatory processes. Indeed, by interfering with NF-κB nuclear translocation VDR agonists attenuate inflammation in several organ cell types such as adipocytes, thyrocytes, cardiomyocytes, in association to a decrease in proinflammatory cytokine production [20, 21, 104, 105].

We have recently reported on the effect of less-hypercalcemic VDR agonist BXL-01-0029 in human skeletal muscle cells under maximal inflammatory stimuli; it decreases CXCL10 secretion with the highest potency versus other current immunosuppressants and specifically deactivates TNFα pathways: JNK phosphorylation is reduced and, quite remarkably in our opinion, NF-κB activation is prevented, while Stat1 activation, downstream of IFNγ, is unaffected. Hence, it appears that BXL-01-0029-induced inhibition of TNFα signal path may be sufficient to significantly decrease CXCL10 secretion by human skeletal muscle cells. This result seems quite intriguing considering the pivotal role of TNFα in muscular inflammation. Accordingly, in human skeletal muscle cells, differently from other cell types, such as human cardiomyocytes, thyrocytes, and renal cells, CXCL10 secretion seems to be essentially dependent on TNFα-driven mechanisms.

As previously addressed, counteracting TNFα signal and, therefore, CXCL10 local accumulation might result in a relevant crumble in the self-enhancing inflammatory loop established between immune infiltrating cells and resident muscular cells. This effect appears even more significant when considering the limited efficacy of the current immunosuppressants in controlling inflammation in IMs.

An additional consequence of TNFα detrimental effects is a possible dysregulation of mitochondrial metabolic pathways occurring in inflammatory muscle diseases [106, 107]: abnormalities in energy regulating paths and deficiencies in glycolytic enzymes have been often observed in IM fibers with more pronounced damages [108, 109]. Even though those mechanisms have still to be fully clarified, myoblasts, immature myofibers, and proinflammatory cytokines, such as TNFα and IL-15, seem the focal point for a cross talk between muscle inflammation and metabolism [34]. So far, targeting proinflammatory muscular cytokines secretion directly in myoblasts may be a quite helpful therapeutic strategy, considering the different possible beneficial effects.

The potential therapeutic application of BXL-01-0029 has been previously shown in nonobese diabetic (NOD) mice, who develop a pathogenesis similar to the human autoimmune type 1 diabetes (T1D), where VDR agonist-induced

block of NF-κB nuclear translocation in pancreatic islets is associated to a significant decrease both in Th1 cell organ infiltration and CXCL10 secretion [110]. We have also previously reported that BXL-01-0029 could be a potential steroid-sparing agent in the current immunosuppressant cocktails used to control inflammation in heart or renal rejection after transplantation [21, 101]. Notably, BXL-01-0029 similarly to elocalcitol, another nonhypercalcemic VDR agonist, does not affect cell viability in several types of organ resident cells and in $CD4^+$ T cells, while significantly decreasing Th1- and Th17-cytokine secretion [20, 101]. Conversely, the majority of immunosuppressants have been designed to reduce the number of immune cells; this specific effect accomplishes the appearance of the well known noxious side effects of immunosuppressive drugs, that is, from metabolic disturbances, to opportunistic infections or tumor development [13–15]. So far, VDR agonists are likely able to control immune reaction acting essentially onto the production of mediators of inflammation, cytokines, and chemokines.

It cannot be ignored that circulating cytokine level are related also to the disease stage, such as acute or chronic, reflecting Th1 or Th2 dominance; in this regard, and in line with a previous study in IM patients in active phase of the diseases [111], we have recently reported that, in sera of subjects at time of diagnosis with IMs CXCL10 is higher than in matched controls, and, importantly, is the highest as compared to some other circulating Th1 cytokines—such as TNFα, IFNγ, IL-8, IL-6, MCP-1, MIP-1β (Th1 type), and IL-10 (Th2 type) [49]. Indeed, CXCL10, as Th1 type chemokine, participates to the early events in inflammatory/immune response and, even more important, is thought to trigger the reaction next to the antigenic challenge [54]. So far, we speculate that pharmacological targeting systemic and especially local muscular CXCL10 production with VDR agonists could result a particularly advantageous approach from the early stage of myositis.

6. Remarks and Conclusion

VDR agonists exert overall repressive effects onto Th1 polarized immune response, which dominates in inflammation, toward a more regulatory Th2 phenotype molecules, which dominate in tolerogenicity. Albeit the current controversy regarding VDR expression in adult skeletal muscle [112], as from our and other data, it appears that VDR agonists exert rapid anti-inflammatory effects directly in skeletal muscle cells. Whether VDR is expressed in skeletal muscle is still a debated issue [1, 112, 113]; the lower basal level in muscle as compared to duodenal cells—widely used as positive control—has been suggested as a possible cause of missing VDR detection in muscle cells [114]. Studies onto contractility and myogenesis showed that VDR is present and engaged in rapid nongenomic vitamin D-induced activation of tyrosine phosphorylation cascade in muscle cells [115]. Several lines of evidence indicate a membrane-associated VDR as the mediator of vitamin D-induced rapid events and, recently, the classical VDR located in caveolae has been shown to mediate vitamin D fast nongenomic signaling in skeletal muscle [114]. The existence of another cell surface receptor for vitamin D, named membrane associated, rapid response steroid binding (MARRS) has been reported in muscle [116].

Albeit the question on whether VDR exists in fully differentiated muscle or plays its pivotal role in myogenesis still is to be clarified, it is undeniable that vitamin D supplementation ameliorates proximal myopathy and muscle pain in patients with severe vitamin D deficiency [2, 117, 118]. Many reports on interventional studies are controversial; however vitamin D supplementation has been reported to improve musculoskeletal function in 12 weeks and reduce the risk of falls after 2 years in institutionalized subjects [5, 6, 119]. Based on their capability to balance immune system homeostasis, without being classical "immunosuppressants," and target local inflammatory mediators at muscular level, VDR ligands appear to be optimal candidates as novel therapeutic agents for IMs. Furthermore, additional benefits of VDR agonists are related to their protective effects against bone-loss, infective pathogens, neoplasies [120–122], all side effects of immunosuppressive agents. Nevertheless, the use of VDR agonists in clinics is generally not pursued and is limited to calcipotriol, a drug applied for psoriasis topic treatment [123].

In fact, despite many advantages, the limit in therapeutic applications of vitamin D and vitamin D analogues undeniably relies on the systemic toxicity often associated with long-term intake: hypercalcemia is the main risk associated to the supraphysiological doses of vitamin D necessary to reach the low local effective concentration [124, 125]. Thus, the introduction of new molecules with immunosuppressive features without causing significant hypercalcemia has been strongly encouraged [126]. For this reason, drug development has been focusing on designing VDR agonists with a distinct separation between immunomodulatory and hypercalcemic potency. The selectivity is function of altered pharmacokinetics in comparison with the natural counterpart but, albeit quite many molecules eliciting much less calcemic effects have been developed, still discrepancies emerge between the therapeutic potential, as set in experimental work, and clinical data [127]. Those frustrating results may also depend on the lack of large and well designed trials.

Molecules with less or none hypercalcemic activity, as BXL-01-0029 or elocalcitol, could be suitable candidates—even as steroid-sparing agents—for inclusion in the therapeutic regimens for IMs. In particular, elocalcitol (150 μg/day P.O.) safety and tolerability in terms of calcemic effect has been proven in double-blind randomized study in 101 postmenopausal osteoporotic women, in placebo-controlled phase IIa (119 enrolled subjects) and follow-on phase IIb (514 patients) trial for the treatment of benign prostate hyperplasia (BPH) [128, 129]. Further studies bridging basic, clinic, and pharmacological researches seem mandatory and local administration strategies could be envisioned for IM treatment to overcome systemic drug intake.

As previously stated, inadequate response to therapy and, consequently, poor outcome are often encountered by IM patients. The major concern in clinics is the absence of internationally validated evaluation criteria to conduct randomized controlled trials [14, 15]: in fact, the few validated assessment tools available provide limited information helpful to patient management. To overcome this limit,

multidisciplinary consortiums, such as the International Myositis Assessment and Clinical Studies Group (IMACS, http://www.niehs.nih.gov/research/resources/imacs/), or Paediatric Rheumatology International Trials Organization (PRINTO, https://www.printo.it/)—including rheumatologists, neurologists, physiatrists dermatologists, and other myositis experts—have been established to develop consensus and standards for the conduct and reporting of myositis [14]. Albeit recent advances in understanding the pathogenesis of myositis are unquestionably important, adequate multicentre trials with validated outcome measures represent a must to be pursued in order to define the best treatment for IMs and give clinical remission as a realistic objective to IM patients.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

References

[1] C. M. Girgis, R. J. Clifton-Bligh, M. W. Hamrick, M. F. Holick, and J. E. Gunton, "The roles of vitamin D in skeletal muscle: form, function, and metabolism," *Endocrine Review*, vol. 34, pp. 33–83, 2013.

[2] L. Ceglia, "Vitamin D and its role in skeletal muscle," *Current Opinion in Clinical Nutrition and Metabolic Care*, vol. 12, no. 6, pp. 628–633, 2009.

[3] E. Giovannucci, "Expanding roles of vitamin D," *The Journal of Clinical Endocrinology and Metabolism*, vol. 94, no. 2, pp. 418–420, 2009.

[4] M. Pfeifer, B. Begerow, H. W. Minne, K. Suppan, A. Fahrleitner-Pammer, and H. Dobnig, "Effects of a long-term vitamin D and calcium supplementation on falls and parameters of muscle function in community-dwelling older individuals," *Osteoporosis International*, vol. 20, no. 2, pp. 315–322, 2009.

[5] L. Flicker, R. J. MacInnis, M. S. Stein et al., "Should older people in residential care receive vitamin D to prevent falls? Results of a randomized trial," *Journal of the American Geriatrics Society*, vol. 53, no. 11, pp. 1881–1888, 2005.

[6] H. A. Bischoff-Ferrari, B. Dawson-Hughes, W. C. Willett et al., "Effect of vitamin D on falls: a meta-analysis," *The Journal of the American Medical Association*, vol. 291, no. 16, pp. 1999–2006, 2004.

[7] T. H. J. Burne, J. J. McGrath, D. W. Eyles, and A. Mackay-Sim, "Behavioural characterization of vitamin D receptor knockout mice," *Behavioural Brain Research*, vol. 157, no. 2, pp. 299–308, 2005.

[8] I. Endo, D. Inoue, T. Mitsui et al., "Deletion of vitamin D receptor gene in mice results in abnormal skeletal muscle development with deregulated expression of myoregulatory transcription factors," *Endocrinology*, vol. 144, no. 12, pp. 5138–5144, 2003.

[9] Y. C. Li, A. E. Pirro, M. Amling et al., "Targeted ablation of the vitamin D receptor: an animal model of vitamin D-dependent rickets type II with alopecia," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 18, pp. 9831–9835, 1997.

[10] A. Windelinckx, G. de Mars, G. Beunen et al., "Polymorphisms in the vitamin D receptor gene are associated with muscle strength in men and women," *Osteoporosis International*, vol. 18, no. 9, pp. 1235–1242, 2007.

[11] C. M. Girgis, R. J. Clifton-Bligh, N. Mokbel, K. Cheng, and J. E. Gunton, "Vitamin D signaling regulates proliferation, differentiation and myotube size in C2C12 skeletal muscle cells," *Endocrinology*, vol. 155, pp. 347–357, 2014.

[12] J. W. Prineas, A. S. Mason, and R. A. Henson, "Myopathy in metabolic bone disease," *British Medical Journal*, vol. 1, pp. 1034–1036, 1965.

[13] M. C. Dalakas, "Immunotherapy of myositis: issues, concerns and future prospects," *Nature Reviews Rheumatology*, vol. 6, no. 3, pp. 129–137, 2010.

[14] F. W. Miller, "New approaches to the assessment and treatment of the idiopathic inflammatory myopathies," *Annals of the Rheumatic Diseases*, vol. 71, no. 2, pp. i82–i85, 2012.

[15] F. C. Ernste and A. M. Reed, "Idiopathic inflammatory myopathies: current trends in pathogenesis, clinical features, and up-to-date treatment recommendations," *Mayo Clinical Proceeding*, vol. 88, pp. 83–105, 2013.

[16] B. Hamilton, "Vitamin D and human skeletal muscle," *Scandinavian Journal of Medicine and Science in Sports*, vol. 20, no. 2, pp. 182–190, 2010.

[17] R. Bouillon, H. Bischoff-Ferrari, and W. Willett, "Vitamin D and health: perspectives from mice and man," *Journal of Bone and Mineral Research*, vol. 23, no. 7, pp. 974–979, 2008.

[18] C. J. Glueck and B. Conrad, "Severe vitamin D deficiency, myopathy and rhabdomyolysis," *North American Journal of Medical Sciences*, vol. 5, pp. 494–495, 2013.

[19] Y. Arnson, H. Amital, and Y. Shoenfeld, "Vitamin D and autoimmunity: new aetiological and therapeutic considerations," *Annals of the Rheumatic Diseases*, vol. 66, no. 9, pp. 1137–1142, 2007.

[20] E. Borgogni, E. Sarchielli, M. Sottili et al., "Elocalcitol inhibits inflammatory responses in human thyroid cells and T cells," *Endocrinology*, vol. 149, no. 7, pp. 3626–3634, 2008.

[21] M. Sottili, L. Cosmi, E. Borgogni et al., "Immunomodulatory effects of BXL-01-0029, a less hypercalcemic vitamin D analogue, in human cardiomyocytes and T cells," *Experimental Cell Research*, vol. 315, no. 2, pp. 264–273, 2009.

[22] M. C. Dalakas, "Medical progress: polymyositis, dermatomyositis, and inclusion-body myositis," *The New England Journal of Medicine*, vol. 325, no. 21, pp. 1487–1498, 1991.

[23] M. C. Dalakas, "Pathophysiology of inflammatory and autoimmune myopathies," *Presse Medicale*, vol. 40, no. 4, pp. e237–e247, 2011.

[24] R. Mantegazza, P. Bernasconi, P. Confalonieri, and F. Cornelio, "Inflammatory myopathies and systemic disorders: a review of immunopathogenetic mechanisms and clinical features," *Journal of Neurology*, vol. 244, no. 5, pp. 277–287, 1997.

[25] C. Grundtman, V. Malmström, and I. E. Lundberg, "Immune mechanisms in the pathogenesis of idiopathic inflammatory myopathies," *Arthritis Research and Therapy*, vol. 9, no. 2, article 208, 2007.

[26] K. Arahata and A. G. Engel, "Monoclonal antibody analysis of mononuclear cells in myopathies. I: quantitation of subsets according to diagnosis and sites of accumulation and demonstration and counts of muscle fibers invaded by T cells," *Annals of Neurology*, vol. 16, no. 2, pp. 193–208, 1984.

[27] A. G. Engel, K. Arahata, and A. Emslie-Smith, "Immune effector mechanisms in inflammatory myopathies," *Research Publications—Association for Research in Nervous and Mental Disease*, vol. 68, pp. 141–157, 1990.

[28] B. de Paepe, K. K. Creus, and J. L. de Bleecker, "Role of cytokines and chemokines in idiopathic inflammatory myopathies," *Current Opinion in Rheumatology*, vol. 21, no. 6, pp. 610–616, 2009.

[29] K. Nagaraju, "Update on immunopathogenesis in inflammatory myopathies," *Current Opinion in Rheumatology*, vol. 13, no. 6, pp. 461–468, 2001.

[30] J.-H. Choi, Y.-E. Park, S.-I. Kim et al., "Differential Immunohistological features of inflammatory myopathies and dysferlinopathy," *Journal of Korean Medical Science*, vol. 24, no. 6, pp. 1015–1023, 2009.

[31] A. Tournadre and P. Miossec, "Chemokines and dendritic cells in inflammatory myopathies," *Annals of the Rheumatic Diseases*, vol. 68, no. 3, pp. 300–304, 2009.

[32] M. C. Dalakas and R. Hohlfeld, "Polymyositis and dermatomyositis," *The Lancet*, vol. 362, no. 9388, pp. 971–982, 2003.

[33] R. Hohlfeld, A. G. Engel, N. Goebels, and L. Behrens, "Cellular immune mechanisms in inflammatory myopathies," *Current Opinion in Rheumatology*, vol. 9, no. 6, pp. 520–526, 1997.

[34] S. Rayavarapu, W. Coley, T. B. Kinder, and K. Nagaraju, "Idiopathic inflammatory myopathies: pathogenic mechanisms of muscle weakness," *Skeletal Muscle*, vol. 3, article 13, 2013.

[35] A. Brunn, K. Zornbach, V. H. Hans, W. F. Haupt, and M. Deckert, "Toll-like receptors promote inflammation in idiopathic inflammatory myopathies," *Journal of Neuropathology and Experimental Neurology*, vol. 3, pp. 855–867, 2012.

[36] I. Lundberg, J. M. Brengman, and A. G. Engel, "Analysis of cytokine expression in muscle in inflammatory myopathies, Duchenne dystrophy, and non-weak controls," *Journal of Neuroimmunology*, vol. 63, no. 1, pp. 9–16, 1995.

[37] B. de Paepe, K. K. Creus, and J. L. de Bleecker, "Chemokines in idiopathic inflammatory myopathies," *Frontiers in Bioscience*, vol. 13, no. 7, pp. 2548–2577, 2008.

[38] D. S. Tews and H. H. Goebel Cytokine, "Expression profile in idiopathic inflammatory myopathies," *Journal of Neuropathology & Experimental Neurology*, vol. 55, pp. 342–347, 1996.

[39] S. Scolletta, M. Colletti, L. di Luigi, and C. Crescioli, "Vitamin D receptor agonists target CXCL10: new therapeutic tools for resolution of inflammation," *Mediators of Inflammation*, vol. 2013, Article ID 876319, 11 pages, 2013.

[40] P. Romagnani and C. Crescioli, "CXCL10: a candidate biomarker in transplantation," *Clinica Chimica Acta*, vol. 413, pp. 1364–1373, 2012.

[41] K. Nagaraju, N. Raben, G. Merritt, L. Loeffler, K. Kirk, and P. Plotz, "A variety of cytokines and immunologically relevant surface molecules are expressed by normal human skeletal muscle cells under proinflammatory stimuli," *Clinical and Experimental Immunology*, vol. 113, no. 3, pp. 407–414, 1998.

[42] B. K. Pedersen, "Edward F. Adolph Distinguished Lecture: muscle as an endocrine organ: IL-6 and other myokines," *The Journal of Applied Physiology*, vol. 107, no. 4, pp. 1006–1014, 2009.

[43] I. Loell and I. E. Lundberg, "Can muscle regeneration fail in chronic inflammation: a weakness in inflammatory myopathies?" *Journal of Internal Medicine*, vol. 269, no. 3, pp. 243–257, 2011.

[44] M. E. DeVries, K. A. Hosiawa, C. M. Cameron et al., "The role of chemokines and chemokine receptors in alloantigen-independent and alloantigen-dependent transplantation injury," *Seminars in Immunology*, vol. 15, no. 1, pp. 33–48, 2003.

[45] M. de Rossi, P. Bernasconi, F. Baggi, R. de Waal Malefyt, and R. Mantegazza, "Cytokines and chemokines are both expressed by human myoblasts: possible relevance for the immune pathogenesis of muscle inflammation," *International Immunology*, vol. 12, no. 9, pp. 1329–1335, 2000.

[46] R. Raju, O. M. Vasconcelos, C. Semino-Mora, R. P. Granger, and M. C. Dalakas, "Expression of interferon-gamma inducible chemokines in the muscles of patients with inclusion body myositis," *Neurology*, vol. 58, p. A390, 2002.

[47] P. Confalonieri, P. Bernasconi, P. Megna, S. Galbiati, F. Cornelio, and R. Mantegazza, "Increased expression of β-chemokines in muscle of patients with inflammatory myopathies," *Journal of Neuropathology and Experimental Neurology*, vol. 59, no. 2, pp. 164–169, 2000.

[48] C. Crescioli, M. Sottili, P. Bonini et al., "Inflammatory response in human skeletal muscle cells: CXCL10 as a potential therapeutic target," *European Journal of Cell Biology*, vol. 91, no. 2, pp. 139–149, 2012.

[49] L. di Luigi, M. Sottili, C. Antinozzi et al., "The vitamin D receptor agonist BXL-01-0029 as a potential new pharmacological tool for the treatment of inflammatory myopathies," *PLoS ONE*, vol. 8, Article ID e77745, 2013.

[50] A. D. Luster and J. V. Ravetch, "Biochemical characterization of a γ interferon-inducible cytokine (IP-10)," *Journal of Experimental Medicine*, vol. 166, no. 4, pp. 1084–1097, 1987.

[51] M. Loetscher, B. Gerber, P. Loetscher et al., "Chemokine receptor specific for IP10 and Mig: structure, function, and expression in activate T-Lymphocytes," *Journal of Experimental Medicine*, vol. 184, no. 3, pp. 963–969, 1996.

[52] M. Loetscher, P. Moetscher, N. Brass, E. Meese, and B. Moser, "Lymphocyte-specific chemokine receptor CXCR3: regulation, chemokine binding and gene localization," *European Journal of Immunology*, vol. 28, pp. 3696–3705, 1998.

[53] S. Qin, J. B. Rottman, P. Myers et al., "The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions," *The Journal of Clinical Investigation*, vol. 101, no. 4, pp. 746–754, 1998.

[54] J. D. Campbell, V. Gangur, F. E. R. Simons, and K. T. HayGlass, "Allergic humans are hyporesponsive to a CXCR3 ligand-mediated Th1 immunity-promoting loop," *The FASEB Journal*, vol. 18, no. 2, pp. 329–331, 2004.

[55] D. Figarella-Branger, M. Civatte, C. Bartoli, and J.-F. Pellissier, "Cytokines, chemokines, and cell adhesion molecules in inflammatory myopathies," *Muscle and Nerve*, vol. 28, no. 6, pp. 659–682, 2003.

[56] L. Frasca, M. Nasso, F. Spensieri et al., "IFN-γ arms human dendritic cells to perform multiple effector functions," *Journal of Immunology*, vol. 180, no. 3, pp. 1471–1481, 2008.

[57] S. Forsgren, L. Renström, C. Purdam, and J. E. Gaida, "TNF-alpha in the locomotor system beyond joints: high degree of involvement in myositis in a rabbit model," *International Journal of Rheumatology*, vol. 2012, Article ID 637452, 11 pages, 2012.

[58] H. Chinoy, F. Salway, S. John et al., "Tumour necrosis factor-α single nucleotide polymorphisms are not independent of HLA class I in UK Caucasians with adult onset idiopathic inflammatory myopathies," *Rheumatology*, vol. 46, no. 9, pp. 1411–1416, 2007.

[59] C. W. Keller, C. Fokken, S. G. Turville et al., "TNF-α induces macroautophagy and regulates MHC class II expression in human skeletal muscle cells," *The Journal of Biological Chemistry*, vol. 286, no. 5, pp. 3970–3980, 2011.

[60] A. R. Baudy, N. Saxena, H. Gordish, E. P. Hoffman, and K. Nagaraju, "A robust in vitro screening assay to identify NF-κB inhibitors for inflammatory muscle diseases," *International Immunopharmacology*, vol. 9, no. 10, pp. 1209–1214, 2009.

[61] T. Sugihara, N. Okiyama, N. Watanabe, N. Miyasaka, and H. Kohsaka, "Interleukin-1 and tumor necrosis factor α blockade treatment of experimental polymyositis in mice," *Arthritis and Reumatism*, vol. 64, pp. 2655–2662, 2012.

[62] A. Tournadre, J.-J. Dubost, and M. Soubrier, "Treatment of inflammatory muscle disease in adults," *Joint Bone Spine*, vol. 77, no. 5, pp. 390–394, 2010.

[63] S. Hodgetts, H. Radley, M. Davies, and M. D. Grounds, "Reduced necrosis of dystrophic muscle by depletion of host neutrophils, or blocking TNFα function with Etanercept in mdx mice," *Neuromuscular Disorders*, vol. 16, no. 9-10, pp. 591–602, 2006.

[64] P. Efthimiou, "Tumor necrosis factor-α in inflammatory myopathies: pathophysiology and therapeutic implications," *Seminars in Arthritis and Rheumatism*, vol. 36, no. 3, pp. 168–172, 2006.

[65] S. Dold, M. E. Justiniano, J. Marquez, and L. R. Espinoza, "Treatment of early and refractory dermatomyositis with infliximab: a report of two cases," *Clinical Rheumatology*, vol. 26, no. 7, pp. 1186–1188, 2007.

[66] T. Hehlgans and K. Pfeffer, "The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: players, rules and the games," *Immunology*, vol. 115, no. 1, pp. 1–20, 2005.

[67] G. C. Suvannavejh, H.-O. Lee, J. Padilla, M. C. Dal Canto, T. A. Barrett, and S. D. Miller, "Divergent roles for p55 and p75 tumor necrosis factor receptors in the pathogenesis of MOG_{35-55}-induced experimental autoimmune encephalomyelitis," *Cellular Immunology*, vol. 205, no. 1, pp. 24–33, 2000.

[68] Y. Abu-Amer and Z. Bar-Shavit, "Impaired bone marrow-derived macrophage differentiation in vitamin D deficiency," *Cellular Immunology*, vol. 151, no. 2, pp. 356–368, 1993.

[69] H. P. Koeffler, H. Reichel, J. E. Bishop, and A. W. Norman, "γ-interferon stimulates production of 1,25-dihydroxyvitamin D_3 by normal human macrophages," *Biochemical and Biophysical Research Communications*, vol. 127, no. 2, pp. 596–603, 1985.

[70] H. Reichel, H. P. Koeffler, J. E. Bishop, and A. W. Norman, "25-hydroxyvitamin D_3 metabolism by lipopolysaccharide-stimulated normal human macrophages," *The Journal of Clinical Endocrinology and Metabolism*, vol. 64, no. 1, pp. 1–9, 1987.

[71] L. J. Dickie, L. D. Church, L. R. Coulthard, R. J. Mathews, P. Emery, and M. F. McDermott, "Vitamin D_3 down-regulates intracellular Toll-like receptor 9 expression and Toll-like receptor 9-induced IL-6 production in human monocytes," *Rheumatology*, vol. 49, no. 8, pp. 1466–1471, 2010.

[72] K. Sadeghi, B. Wessner, U. Laggner et al., "Vitamin D_3 down-regulates monocyte TLR expression and triggers hyporesponsiveness to pathogen-associated molecular patterns," *European Journal of Immunology*, vol. 36, no. 2, pp. 361–370, 2006.

[73] D. M. Provvedini, C. D. Tsoukas, L. J. Deftos, and S. C. Manolagas, "1,25-dihydroxyvitamin D_3 receptors in human leukocytes," *Science*, vol. 221, no. 4616, pp. 1181–1183, 1983.

[74] F. Baeke, H. Korf, L. Overbergh et al., "Human T lymphocytes are direct targets of 1,25-dihydroxyvitamin D_3 in the immune system," *The Journal of Steroid Biochemistry and Molecular Biology*, vol. 121, no. 1-2, pp. 221–227, 2010.

[75] A. K. Bhalla, E. P. Amento, B. Serog, and L. H. Glimcher, "1,25-dihydroxyvitamin D_3 inhibits antigen-induced T cell activation," *Journal of Immunology*, vol. 133, no. 4, pp. 1748–1754, 1984.

[76] J. M. Lemire, D. C. Archer, L. Beck, and H. L. Spiegelberg, "Immunosuppressive actions of 1,25-dihydroxyvitamin D_3: preferential inhibition of Th1 functions," *The Journal of Nutrition*, vol. 125, no. 6, pp. 1704S–1708S, 1995.

[77] F. Matter, S. Smiroldo, F. Galbiati et al., "Inhibition of Th1 development and treatment of chronic-relapsing experimental allergic encephalomyelitis by a non-hypercalcemic analogue of 1, 25-dihydroxyvitamin D_3," *European Journal of Immunology*, vol. 30, pp. 498–508, 2000.

[78] I. Alroy, T. L. Towers, and L. P. Freedman, "Transcriptional repression of the interleukin-2 gene by vitamin D_3: direct inhibition of NFATp/AP-1 complex formation by a nuclear hormone receptor," *Molecular and Cellular Biology*, vol. 15, no. 10, pp. 5789–5799, 1995.

[79] A. Takeuchi, G. S. Reddy, T. Kobayashi, T. Okano, J. Park, and S. Sharma, "Nuclear factor of activated T cells (NFAT) as a molecular target for 1α,25-dihydroxyvitamin D_3-mediated effects," *Journal of Immunology*, vol. 160, no. 1, pp. 209–218, 1998.

[80] M. Cippitelli and A. Santoni, "Vitamin D_3: a transcriptional modulator of the interferon-gamma gene," *European Journal of Immunology*, vol. 28, pp. 3017–3030, 1998.

[81] M. T. Cantorna, W. D. Woodward, C. E. Hayes, and H. F. DeLuca, "1,25-dihydroxyvitamin D_3 is a positive regulator for the two anti- encephalitogenic cytokines TGF-β1 and IL-4," *Journal of Immunology*, vol. 160, no. 11, pp. 5314–5319, 1998.

[82] M. D. Griffin, N. Xing, and R. Kumar, "Vitamin D and its analogs as regulators of immune activation and antigen presentation," *Annual Review of Nutrition*, vol. 23, pp. 117–145, 2003.

[83] Q. Li and I. M. Verma, "NF-κB regulation in the immune system," *Nature Reviews Immunology*, vol. 2, no. 10, pp. 725–734, 2002.

[84] E. Hyppönen, E. Läärä, A. Reunanen, M.-R. Järvelin, and S. M. Virtanen, "Intake of vitamin D and risk of type 1 diabetes: a birth-cohort study," *The Lancet*, vol. 358, no. 9292, pp. 1500–1503, 2001.

[85] D. D. Branisteanu, M. Waer, H. Sobis, S. Marcelis, M. Vandeputte, and R. Bouillon, "Prevention of murine experimental allergic encephalomyelitis: cooperative effects of cyclosporine and 1 α,25-$(OH)_2D_3$," *Journal of Neuroimmunology*, vol. 61, no. 2, pp. 151–160, 1995.

[86] M. K. Racke, S. Dhib-Jalbut, B. Cannella, P. S. Albert, C. S. Raine, and D. E. McFarlin, "Prevention and treatment of chronic relapsing experimental allergic encephalomyelitis by transforming growth factor-β1," *Journal of Immunology*, vol. 146, no. 9, pp. 3012–3017, 1991.

[87] S. Gregori, M. Casorati, S. Amuchastegui, S. Smiroldo, A. M. Davalli, and L. Adorini, "Regulatory T cells induced by 1α,25-dihydroxyvitamin D_3 and mycophenolate mofetil treatment mediate transplantation tolerance," *Journal of Immunology*, vol. 167, no. 4, pp. 1945–1953, 2001.

[88] M. D. Griffin, W. Lutz, V. A. Phan, L. A. Bachman, D. J. McKean, and R. Kumar, "Dendritic cell modulation by 1α,25 dihydroxyvitamin D_3 and its analogs: a vitamin D receptor-dependent pathway that promotes a persistent state of immaturity in vitro and in vivo," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 12, pp. 6800–6805, 2001.

[89] J. Wang, M. Nuite, and T. E. McAlindon, "Association of estrogen and aromatase gene polymorphisms with systemic lupus erythematosus," *Lupus*, vol. 19, no. 6, pp. 734–740, 2010.

[90] J.-C. Souberbielle, J.-J. Body, J. M. Lappe et al., "Vitamin D and musculoskeletal health, cardiovascular disease, autoimmunity and cancer: recommendations for clinical practice," *Autoimmunity Reviews*, vol. 9, no. 11, pp. 709–715, 2010.

[91] E. M. Colin, P. S. Asmawidjaja, J. P. van Hamburg et al., "1,25-dihydroxyvitamin D_3 modulates Th17 polarization and interleukin-22 expression by memory T cells from patients with early rheumatoid arthritis," *Arthritis and Rheumatism*, vol. 62, no. 1, pp. 132–142, 2010.

[92] A. Boonstra, F. J. Barrat, C. Crain, V. L. Heath, H. F. J. Savelkoul, and A. O'Garra, "1α,25-dihydroxyvitamin D_3 has a direct effect on naïve $CD4^+$ T cells to enhance the development of Th2 cells," *Journal of Immunology*, vol. 167, no. 9, pp. 4974–4980, 2001.

[93] E. van Etten and C. Mathieu, "Immunoregulation by 1,25-dihydroxyvitamin D_3: basic concepts," *The Journal of Steroid Biochemistry and Molecular Biology*, vol. 97, no. 1-2, pp. 93–101, 2005.

[94] A. W. Pedersen, K. Holmstrøm, S. S. Jensen et al., "Phenotypic and functional markers for 1α,25-dihydroxyvitamin D_3-modified regulatory dendritic cells," *Clinical and Experimental Immunology*, vol. 157, no. 1, pp. 48–59, 2009.

[95] S. Joshi, L.-C. Pantalena, X. K. Liu et al., "1,25-dihydroxyvitamin D_3 ameliorates Th17 autoimmunity via transcriptional modulation of interleukin-17A," *Molecular and Cellular Biology*, vol. 31, no. 17, pp. 3653–3669, 2011.

[96] A.-L. Khoo, L. Y. A. Chai, H. J. P. M. Koenen et al., "Regulation of cytokine responses by seasonality of vitamin D status in healthy individuals," *Clinical and Experimental Immunology*, vol. 164, no. 1, pp. 72–79, 2011.

[97] J. Fritsche, K. Mondal, A. Ehrnsperger, R. Andreesen, and M. Kreutz, "Regulation of 25-hydroxyvitamin D3-1 alpha-hydroxylase and production of 1 alpha,25-dihydroxyvitamin D3 by human dendritic cells," *Blood*, vol. 102, no. 9, pp. 3314–3316, 2003.

[98] S. Chen, G. P. Sims, X. X. Chen, Y. Y. Gu, S. Chen, and P. E. Lipsky, "Modulatory effects of 1,25-dihydroxyvitamin D_3 on human B cell differentiation," *Journal of Immunology*, vol. 179, no. 3, pp. 1634–1647, 2007.

[99] K. Geldmeyer-Hilt, G. Heine, B. Hartmann, R. Baumgrass, A. Radbruch, and M. Worm, "1,25-dihydroxyvitamin D_3 impairs NF-κB activation in human naïve B cells," *Biochemical and Biophysical Research Communications*, vol. 407, no. 4, pp. 699–702, 2011.

[100] L. Adorini, "Intervention in autoimmunity: the potential of vitamin D receptor agonists," *Cellular Immunology*, vol. 233, no. 2, pp. 115–124, 2005.

[101] C. Sagrinati, M. Sottili, B. Mazzinghi et al., "Comparison between VDR analogs and current immunosuppressive drugs in relation to CXCL10 secretion by human renal tubular cells," *Transplant International*, vol. 23, no. 9, pp. 914–923, 2010.

[102] C. Crescioli, L. Cosmi, E. Borgogni et al., "Methimazole inhibits CXC chemokine ligand 10 secretion in human thyrocytes," *Journal of Endocrinology*, vol. 195, no. 1, pp. 145–155, 2007.

[103] C. Crescioli, R. Squecco, L. Cosmi et al., "Immunosuppression in cardiac graft rejection: a human in vitro model to study the potential use of new immunomodulatory drugs," *Experimental Cell Research*, vol. 314, no. 6, pp. 1337–1350, 2008.

[104] S. J. Mutt, T. Karhu, S. Lehtonen et al., "Inhibition of cytokine secretion from adipocytes by 1,25-dihydroxyvitamin D_3 via the NF-κB pathway," *The FASEB Journal*, vol. 26, no. 11, pp. 4400–4407, 2012.

[105] Y. Chen, J. Kong, T. Sun et al., "1,25-dihydroxyvitamin D_3 suppresses inflammation-induced expression of plasminogen activator inhibitor-1 by blocking nuclear factor-κB activation," *Archives of Biochemistry and Biophysics*, vol. 507, no. 2, pp. 241–247, 2011.

[106] P. Temiz, C. C. Weihl, and A. Pestronk, "Inflammatory myopathies with mitochondrial pathology and protein aggregates," *Journal of the Neurological Sciences*, vol. 278, no. 1-2, pp. 25–29, 2009.

[107] J. M. Schröder and M. Molnar, "Mitochondrial abnormalities and peripheral neuropathy in inflammatory myopathy, especially inclusion body myositis," *Molecular and Cellular Biochemistry*, vol. 174, no. 1-2, pp. 277–281, 1997.

[108] Ó. Miró, J. Casademont, J. M. Grau, D. Jarreta, Á. Urbano-Márquez, and F. Cardellach, "Histological and biochemical assessment of mitochondrial function in dermatomyositis," *British Journal of Rheumatology*, vol. 37, no. 10, pp. 1047–1053, 1998.

[109] M. I. Alhatou, J. T. Sladky, O. Bagasra, and J. D. Glass, "Mitochondrial abnormalities in dermatomyositis: characteristic pattern of neuropathology," *Journal of Molecular Histology*, vol. 35, no. 6, pp. 615–619, 2004.

[110] S. Gregori, N. Giarratana, S. Smiroldo, M. Uskokovic, and L. Adorini, "A 1α,25-dihydroxyvitamin D_3 analog enhances regulatory T-cells and arrests autoimmune diabetes in NOD mice," *Diabetes*, vol. 51, no. 5, pp. 1367–1374, 2002.

[111] P. Szodoray, P. Alex, N. Knowlton et al., "Idiopathic inflammatory myopathies, signified by distinctive peripheral cytokines, chemokines and the TNF family members B-cell activating factor and a proliferation inducing ligand," *Rheumatology*, vol. 49, no. 10, pp. 1867–1877, 2010.

[112] Y. Wang and H. F. DeLuca, "Is the vitamin D receptor found in muscle?" *Endocrinology*, vol. 152, no. 2, pp. 354–363, 2011.

[113] H. A. Bischoff, M. Borchers, F. Gudat et al., "In situ detection of 1,25-dihydroxyvitamin D_3 receptor in human skeletal muscle tissue," *Histochemical Journal*, vol. 33, no. 1, pp. 19–24, 2001.

[114] C. Buitrago, V. Gonzalez Pardo, and R. Boland, "Role of VDR in 1α, 25-dihydroxyvitamin D_3 -dependent non-genomic activation of MAPKs, Src and Akt in skeletal muscle cells," *Journal of Steroid Biochemistry & Molecular Biology*, vol. 136, pp. 125–130, 2013.

[115] R. L. Boland, "VDR activation of intracellular signaling pathways in skeletal muscle," *Molecular and Cellular Endocrinology*, vol. 347, no. 1-2, pp. 11–16, 2011.

[116] R. C. Khanal and I. Nemere, "The ERp57/GRp58/1,25D_3-MARRS receptor: multiple functional roles in diverse cell systems," *Current Medicinal Chemistry*, vol. 14, no. 10, pp. 1087–1093, 2007.

[117] R. J. Menaker, P. J. M. Ceponis, and N. L. Jones, "Helicobacter pylori induces apoptosis of macrophages in association with alterations in the mitochondrial pathway," *Infection and Immunity*, vol. 72, no. 5, pp. 2889–2898, 2004.

[118] S. E. Mercer, D. Z. Ewton, X. Deng, S. Lim, T. R. Mazur, and E. Friedman, "Mirk/Dyrk1B mediates survival during the differentiation of C2C12 myoblasts," *The Journal of Biological Chemistry*, vol. 280, no. 27, pp. 25788–25801, 2005.

[119] C. M. Girgis, R. J. Clifton-Bligh, N. Turner, S. L. Lau, and J. E. Gunton, "Effects of vitamin D in skeletal muscle: falls, strength, athletic performance and insulin sensitivity," *Clinical Endocrinology*, vol. 80, pp. 169–181, 2014.

[120] H.-U. Stempfle, C. Werner, U. Siebert et al., "The role of tacrolimus (FK506)-based immunosuppression on bone mineral density and bone turnover after cardiac transplantation: a prospective, longitudinal, randomized, double-blind trial with calcitriol," *Transplantation*, vol. 73, no. 4, pp. 547–552, 2002.

[121] M. T. Cantorna, D. A. Hullett, C. Redaelli et al., "1,25-dihydroxyvitamin D_3 prolongs graft survival without compromising host resistance to infection or bone mineral density," *Transplantation*, vol. 66, no. 7, pp. 828–831, 1998.

[122] G. Picotto, A. C. Liaudat, L. Bohl, and N. T. Talamoni, "Molecular aspects of vitamin D anticancer activity," *Cancer Investigation*, vol. 30, pp. 604–614, 2012.

[123] L. J. Scott, C. J. Dunn, and K. L. Goa, "Calcipotriol ointment: a review of its use in the management of psoriasis," *American Journal of Clinical Dermatology*, vol. 2, no. 2, pp. 95–120, 2001.

[124] M. di Rosa, M. Malaguarnera, F. Nicoletti, and L. Malaguarnera, "Vitamin D_3: a helpful immuno-modulator," *Immunology*, vol. 134, no. 2, pp. 123–139, 2011.

[125] D. B. Endres, "Investigation of hypercalcemia," *Clinical Biochemistry*, vol. 45, pp. 954–963, 2012.

[126] T. Takahashi and K. Morikawa, "Vitamin D receptor agonists: opportunities and challenges in drug discovery," *Current Topics in Medicinal Chemistry*, vol. 6, no. 12, pp. 1303–1316, 2006.

[127] S. J. Steddon, N. J. Schroeder, and J. Cunningham, "Vitamin D analogues: how do they differ and what is their clinical role?" *Nephrology Dialysis Transplantation*, vol. 16, no. 10, pp. 1965–1967, 2001.

[128] G. D. Zhu, "Vitamin D analogs currently on the market and in development," in *Why Does Vitamin D Matter?* vol. 26, pp. 57–82, Bentham Science Publisher, 2013.

[129] D. Feldman, J. Wesley Pike, and J. S. Adams, *Vitamin D*, Academic Press, London, UK, 3rd edition, 2011.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 879703, 8 pages
http://dx.doi.org/10.1155/2014/879703

Research Article

7-Tesla Magnetic Resonance Imaging Precisely and Noninvasively Reflects Inflammation and Remodeling of the Skeletal Muscle in a Mouse Model of Antisynthetase Syndrome

Clara Sciorati,[1] Antonio Esposito,[2,3] Lara Campana,[1] Tamara Canu,[2] Antonella Monno,[1] Anna Palmisano,[2] Francesco De Cobelli,[2,3] Alessandro Del Maschio,[2,3] Dana P. Ascheman,[4] Angelo A. Manfredi,[1,3] and Patrizia Rovere-Querini[1,3]

[1] *Division of Regenerative Medicine, Stem Cells & Gene Therapy, San Raffaele Scientific Institute, Via Olgettina 60, 20132 Milan, Italy*
[2] *Department of Radiology and Preclinical Imaging Facility, San Raffaele Scientific Institute, Via Olgettina 60, 20132 Milan, Italy*
[3] *School of Medicine, Vita-Salute San Raffaele University, Via Olgettina 58, Milan, Italy*
[4] *University of Miami Miller School of Medicine, Miami, FL 33136, USA*

Correspondence should be addressed to Patrizia Rovere-Querini; rovere.patrizia@hsr.it

Received 25 January 2014; Accepted 4 April 2014; Published 5 May 2014

Academic Editor: Dario Coletti

Inflammatory myopathies comprise heterogeneous disorders. Their etiopathogenesis is poorly understood, because of the paucity of informative experimental models and of approaches for the noninvasive study of inflamed tissues. Magnetic resonance imaging (MRI) provides information about the state of the skeletal muscle that reflects various facets of inflammation and remodeling. This technique has been scarcely used in experimental models of inflammatory myopathies. We characterized the performance of MRI in a well-established mouse model of myositis and the antisynthetase syndrome, based on the immunization of wild-type mice with the amino-terminal fragment of histidyl-tRNA synthetase (HisRS). Over an eight-week period following myositis induction, MRI enabled precise identification of pathological events taking place in muscle tissue. Areas of edema and of active inflammation identified by histopathology paralleled muscle modifications detected noninvasively by MRI. Muscles changes were chronologically associated with the establishment of autoimmunity, as reflected by the development of anti-HisRS antibodies in the blood of immunized mice. MR imaging easily appreciated muscle damage and remodeling even if actual disruption of myofiber integrity (as assessed by serum concentrations of creatinine phosphokinase) was limited. Thus, MR imaging represents an informative and noninvasive analytical tool for studying *in vivo* immune-mediated muscle involvement.

1. Introduction

Inflammatory myopathies (IM) comprise a group of heterogeneous muscle diseases that share key common characteristics including in particular muscle weakness, low endurance [1], tissue infiltration by inflammatory cells [2–4], and myofiber necrosis/regeneration with an increase of creatine phosphokinase (CPK) serum levels during acute phases of the disease [5]. The presence of autoantibodies targeting ubiquitous intracellular proteins involved in gene transcription or protein synthesis and translocation [6] highlights an autoimmune origin of the disease. Autoantibodies against histidyl-tRNA synthetase (HisRS, also called Jo-1) are particularly well-studied [7, 8], and their serum level correlates with various measures of disease activity [9].

The pathogenesis of IM is jet poorly understood. Animal models that fully reproduce the various features of human disease are needed [10]. Myositis induced upon immunization with HisRS appears particularly informative, since it reproduces both the break of tolerance towards selected autoantigens and specific combined inflammatory involvement of the skeletal muscle and lung that are hallmarks of the human antisynthetase syndrome [11, 12]. Despite the insight provided by such models, the application of noninvasive

methods for *in vivo* monitoring of disease activity, such as magnetic resonance imaging (MRI), has been lacking.

Magnetic resonance imaging (MRI) is a powerful and informative technique to investigate soft tissues, with the ability to noninvasively characterize parenchymal changes occurring in patients with myositis. Imaging has traditionally had an ancillary role in the diagnosis of myositis and inflammatory myopathies. Routinely, the MRI protocol includes T1-weighted images and fluid-sensitive sequences such as short tau inversion recovery (STIR), providing qualitative information about muscle tone, fat infiltration, and muscle edema [13]. Transaxial and coronal sections of the shoulders and thighs are usually obtained on each patient. T1-weighted images allow to assess the muscle thickness/mass and to score the degree of fatty infiltration; fluid-sensitive sequences detect the presence of edema [13]. Routine MRI performed with T1-weighted and STIR sequences is more sensitive but less specific than biopsy in diagnosis; it is advantageous for optimizing efficacy of classical diagnostic procedures [14]. Its importance is progressively growing, since it allows to noninvasively characterize the distribution and pattern of parenchymal changes and to monitor the disease progression, which has important implications for treatment [13, 15].

In the mouse, MRI has been used after tissue injury induced by maximal lengthening contractions [16] and in experimental models of skeletal muscle dystrophy (*mdx* and dysferlin-deficient mice) to assess disease progression [17]. Foci of high intensity signal in T2-weighted images correspond to dystrophic lesions in *mdx* mice [18, 19], while changes in gadofluorine enhancement were identified in dysferlin-deficient animals [20]. Recently, MRI has also been used in C57BL/6 mice to assess the specific features of the homeostatic response of healthy muscles to acute sterile injury induced by cardiotoxin (CTX) [21]. Specifically, T2 mapping and diffusion-tensor imaging (DTI) provide useful information on the extent of myofibril necrosis and of leukocyte infiltration as well as on the kinetics of regeneration [21]. Here we show that MRI, including advanced quantitative techniques as T2 mapping and DTI, is a useful tool to assess the inflammatory changes and the tissue remodeling associated with autoimmune myositis in an experimental model of HisRS-induced myositis. Architectural changes in the tissue organization were temporally linked to muscle damage and to the establishment of the specific autoantibody response.

2. Materials and Methods

2.1. Animal Model and Study Design. Sterile injury by CTX injection and immunization experiments was carried out using C57BL/6J wild-type mice (Charles River, Wilmington, MA, USA) aged 8–10 weeks. Animals were housed in the pathogen-free facility at our institution and treated in accordance with the European Community guidelines and with the approval of the Institutional Ethical Committee (IACUC number 512). For sterile injury, animals were anesthetized by intraperitoneal injection of tribromoethanol (Avertin) at a dose of 250 mg/Kg and *tibialis anterior* (TA) muscles were injected with CTX (50 μL, 10 μM final concentration, *Naja mossambica mossambica*, Sigma-Aldrich) in phosphate buffer solution (PBS) using an insulin needle (3/10 cc Insulin Syringe from Becton-Dickinson, Franklin Lakes, NJ, USA). Vehicle-treated mice received PBS alone (50 μL). For HisRS immunization, *gastrocnemius* (GS) muscles were injected with a recombinant protein fragment corresponding to the amino-terminal portion (residues 1–151) of the murine HisRS molecule produced as a maltose binding protein and characterized as described in [11]. Immunization was carried out using a mixture of the antigen (4 mg/mL) and incomplete Freund's adjuvant (IFA; 1 : 1, vol : vol; 100 μL/mouse). Vehicle-treated mice received a mixture of PBS and IFA. Independent cohorts (6 mice/group) were monitored by MRI after myositis induction, sham treatment (IFA/PBS), or sterile CTX injury at various time points: times 0 and 7, 14, 28, or 56 days after HisRS immunization and times 0, 1, 3, 7, 10, 15, and 30 days after CTX injection. Table 1 shows experimental timing for the two different models of damage.

2.2. MRI. MRI studies were performed on a 7T preclinical magnetic resonance scanner (Bruker, BioSpec 70/30 USR, Paravision 5.0, Germany), equipped with 450/675 mT/m gradients (slow rate: 3400–4500 T/m/s; rise time: 140 μs). A phased-array rat-heart coil with four internal preamplifiers was used as receiver, coupled with a 72 mm linear-volume coil as transmitter. Mice were under general anesthesia obtained by 1,5–2% isoflurane (Forane, Abbott) vaporized in 100% oxygen (flow: 1 l/min), in prone position, with the right leg fixed in the center of the coil. Breathing and body temperature were monitored during MRI (SA Instruments, Inc., Stony Brook, NY, USA) and maintained around 30 breaths per minute and 37°C, respectively. After positioning in the magnet isocenter, a fieldmap based shimming (MAPSHIM software package, Paravision-5.0, Bruker; Germany) was performed to optimize B0 field homogeneity. MRI parameters were used to assess skeletal muscle that included T2-relaxation time (T2-rt), evaluated by T2 mapping, and fractional anisotropy (F.A.), evaluated by diffusion tensor imaging (DTI). Muscle T2 maps were obtained using a multislice-multiecho (MSME) sequence with fat suppression (repetition time = 1938 ms; 16 echo times = 10.73/171.68 ms; field-of-view = 20 × 20 mm; matrix = 256 × 256; spatial resolution = 0.078 × 0.078 mm/pixel; NSA = 4) acquired on axial plane (10 slices; thickness = 1 mm; gap = 0 mm). DTI data were obtained using a SpinEcho-EPI sequence (DTI-Epi) with 30 diffusion gradient directions (repetition time = 3750 ms; echo time = 33 ms; b-values for direction = 0 sec/mm^2–700 sec/mm^2; diffusion gradient duration = 4 ms; diffusion gradient separation = 20 ms; NSA = 2). DTI-Epi sequence shared the same geometrical features (field-of-view = 30 × 30 mm; matrix = 128 × 128; spatial resolution = 0.234 × 0.234 mm/pixel; 10 slices; slice thickness = 1 mm; gap = 0 mm).

2.3. Image Analysis. MRI postprocessing was performed with Paravision-5.0 software (Bruker). Average ADC, F.A., and T2-rt values were obtained from the regions of interest (ROIs) of five subsequent slices placed both on TA (CTX-injured

Table 1: Experimental settings and timing of evaluations.

	CPK and autoantibodies	MRI	Histopathology
Autoimmunity (HisRS immunization)	0, 7, 14, 28, 42, 56	0, 7, 14, 28, 42, 56	0, 7, 14, 28, 56
Sterile injury (CTX injection)		0, 1, 3, 5, 7, 10, 15, 30	0, 1, 3, 5, 10, 30

muscle) and on GS (HIsRS-injected muscle) muscles of each mouse at each time point.

2.4. Autoantibodies. Anti-HisRS IgG antibodies in the serum were measured using standard solid-phase enzyme-linked immunosorbent assay (ELISA) as described [11, 12]. Briefly ninety-six-well plates (Sigma) were coated with recombinant murine synthetic HisRS N-terminal fragment sequence (0.1 μg/mL) and incubated overnight at 4°C. After blocking with PBS containing 1% bovine serum albumin (BSA), serum at various dilutions in PBS containing 1% BSA was added for 2 hours. Following incubation with HRP-conjugated goat anti-mouse IgG (Sigma), the enzymatic reaction was visualized using 3,3′,5,5′-tetramethylbenzidine (Sigma) and terminated by H_2SO_4 addiction prior to spectrophotometric assessment at 450 nm using a microplate reader (Biorad, Hercules, CA) [11, 12].

2.5. Histological Analysis and Image Acquisition. Animals were sacrificed by cervical dislocation and muscles were dissected, immediately frozen in liquid N_2-cooled isopentane, and conserved at −80°C until analysis. For histological studies, 8 μm serial muscle sections were obtained and stained in hematoxylin and eosin (H&E) following standard procedures. At least 10 sections along the entire muscle length were collected and stained. An expert blinded pathologist evaluated samples assigning a 0-to-5 score (0: healthy muscle and 5: maximum inflammatory infiltration), based on the degree of inflammatory cells identified. For image acquisition, we employed a microscope Nikon Eclipse 55i microscope (Nikon, Tokyo, Japan). Images were captured with Digital Sight DS-5 M digital camera (Nikon) using Lucia G software (Laboratory Imaging, Prague, Czech). Linear adjustments of images were done using Adobe Photoshop CS4.

2.6. CPK Activity. Serum CPK levels were measured in blood samples obtained from the tail vein using an indirect commercially available colorimetric assay according to the manufacturer's instructions (Randox, UK).

2.7. Statistical Analysis. All values were expressed as mean ± S.E.M. All data were analyzed using an unpaired two-tailed Student's t-test for comparisons between two groups. P values lower than 5% ($P < 0.05$) were considered statistically significant.

3. Results

We analyzed changes in the normal architecture of mouse skeletal muscle by multiparametric MRI comparing features associated with the homeostatic response to sterile tissue injury with those associated with the immune-mediated skeletal muscle remodeling characterizing a model of antigen-induced experimental myositis. 10-week-old wild-type mice were injected in GS muscle at day 0 with a synthetic sequence corresponding to the amino-terminal portion of the murine HisRS autoantigen in the presence of adjuvant (IFA). Mice injected with IFA alone (sham-treated) served as controls. Autoantibody induction, CPK levels, histopathological abnormalities, and imaging evidence of muscle inflammation/remodeling were verified at various time points (see Table 1). Anti-HisRS IgG antibodies were detectable by seven days after immunization and increased progressively until day 28, at which point their levels began to decrease. (Figure 1(a)). Two months after a single immunization, antibodies were still significantly higher than before treatment or than in sham-treated mice that never develop detectable anti-HisRS antibodies. Autoimmunity establishment and production of autoantibodies correlate with myofibers necrosis, as assessed by the serum CPK activity which reflects the disruption of sarcolemma integrity: in fact CPK levels were significantly higher in immunized mice starting from two weeks to six weeks after immunization. CPK levels in sham-treated animals remained at background levels, indicating that intramuscular injection per se is not responsible for enzyme elevation (Figure 1(b)).

In animal cohorts studied by the noninvasive technique of MRI, tibial and peroneal bones can be easily identified in noninjured healthy wild-type animal legs (Figure 2(a), yellow arrows) and provide anatomical landmarks for location of TA and GS muscles. Skeletal muscle has intermediate signal intensity on all MR pulse sequences, with T2-relaxation time images (T2-rt) that are rather homogeneous (Figure 2(a), red arrows).

Muscles of immunized mice demonstrated a significant increase in the T2-rt values by two weeks after immunization (Figure 2(b)), that is, at a time in which CPK levels revealed ongoing myofibers necrosis (Figure 1(b)). T2-rt values remained significantly higher throughout the observation period that lasted until 8 weeks after immunization. Interestingly, and in sharp contrast with the characteristics of the T2 mapping in acutely injured muscles (see Figure 3), alterations detected by MRI appeared to be restricted to the regions at the very periphery of leg muscles, preferentially involving the perimuscular connective tissues (Figure 2(a)). In contrast, T2-rt values of sham-treated mice remained very stable after

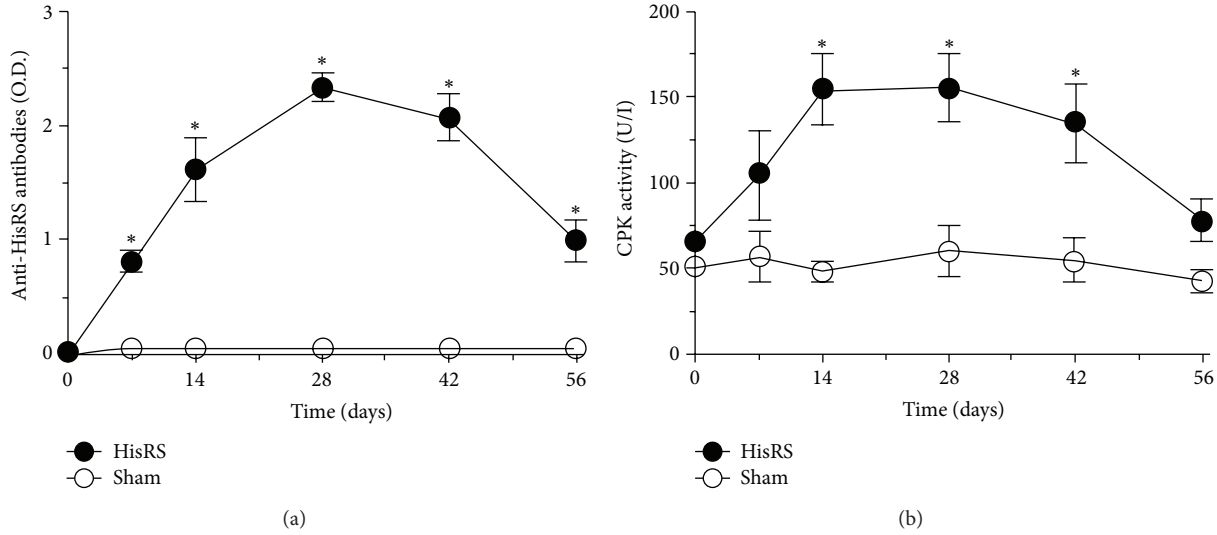


Figure 1: *Anti-HisRS autoantibody induction is associated with sustained muscle damage.* Assessment of anti-HisRS IgG antibodies (a) and CPK activity (b) in serum retrieved before treatment and at various times after immunization. $n \geq 6$; * statistically different from sham-treated mice; $P < 0.05$.

intramuscular injection. Of interest, DTI assessment revealed that F.A. values were substantially stable after immunization and did not differ from those of sham-treated animals (Figure 2(d)). We then verified the histological correlates of the results, observing that tissues of immunized animals undergo a preferential fragmentation of perimysial connective tissue, which is characterized from intense infiltration by mononuclear cells. Edema with limited evidence of necrosis and myofiber atrophy most frequently occurred in proximity to inflamed connective tissue. In contrast, inflammatory involvement of endomysial and perivascular regions was uncommon, with only some degree of muscle regeneration. (Figure 2(a)). Extending these observations, we assessed the link between T2-rt values of immunized mice at various time points and a tissue histological score for inflammation (see the Materials and Methods section for details). T2-rt values and the histological damage/infiltration score were significantly correlated ($r = 0.959$; $P < 0.005$, Figure 2(c)), suggesting that T2-rt values accurately reflect perimysial inflammation.

As an internal control for ongoing muscle remodeling, we studied in parallel the homeostatic response to sterile injury by CTX, an agent that induces transient muscle necrosis associated with well-characterized reversible alterations detectable by multiparametric MRI ([21] and Figure 3). T2-rt values 1 day after sterile injury were significantly higher than those of sham-treated mice and similar to those observed in mice with autoimmune myositis at later time points (compare Figure 3(b) with Figure 2(b)). Kinetic was remarkably different, since in CTX-injured mice T2-rt values dropped to background levels ten days after treatment and remained stable thereafter (Figure 3(b)). Parallel histopathological study of muscle tissue obtained from mice sacrificed after imaging revealed that damage resulted in rapid and massive death of myofibers by day 1, with subsequent infiltration of the tissue by inflammatory cells and appearance of small regenerating/centronucleated fibers by day 5 after injection. At day 10, necrosis and inflammation were hardly apparent, while extensive regeneration was taking place (Figure 3(a)). In contrast, F.A. values dropped 24 hours after injury, possibly because of the simultaneous necrosis of myofibers that abruptly lose their architecture in response to CTX (Figure 3(c)). With time, F.A. values returned to normal in conjunction with the successful regeneration of the tissue. Similarly, F.A. values remained relatively stable in HRS-induced myositis, that is, associated with fairly limited necrosis and regeneration (Figure 2(d)), further supporting the relationship between F.A. values and the state of muscle repair.

4. Discussion

IM are heterogeneous disorders, histologically characterized by muscle inflammation with the presence of autoreactive lymphocytes, fiber degeneration, and overexpression of MHC class I. Common clinical features are muscle weakness, serum autoantibodies, and elevated muscle enzyme [7, 8]. Recent data suggest not only that both innate immunity and adaptive immunity are involved in the IM pathogenesis but also that intrinsic muscle defects (such as metabolic defects and endoplasmic reticulum stress and autophagy and hypoxia) may contribute to damage [1]. The link between remodeling of skeletal muscle and autoimmune responses, which typically target ubiquitous expressed autoantigens, is extremely difficult to study, as the events underlying reduced muscle strength and endurance [8, 22]. The availability of informative animal models is thus crucial [23]. Traditionally, the assessment of ongoing muscle inflammatory involvement

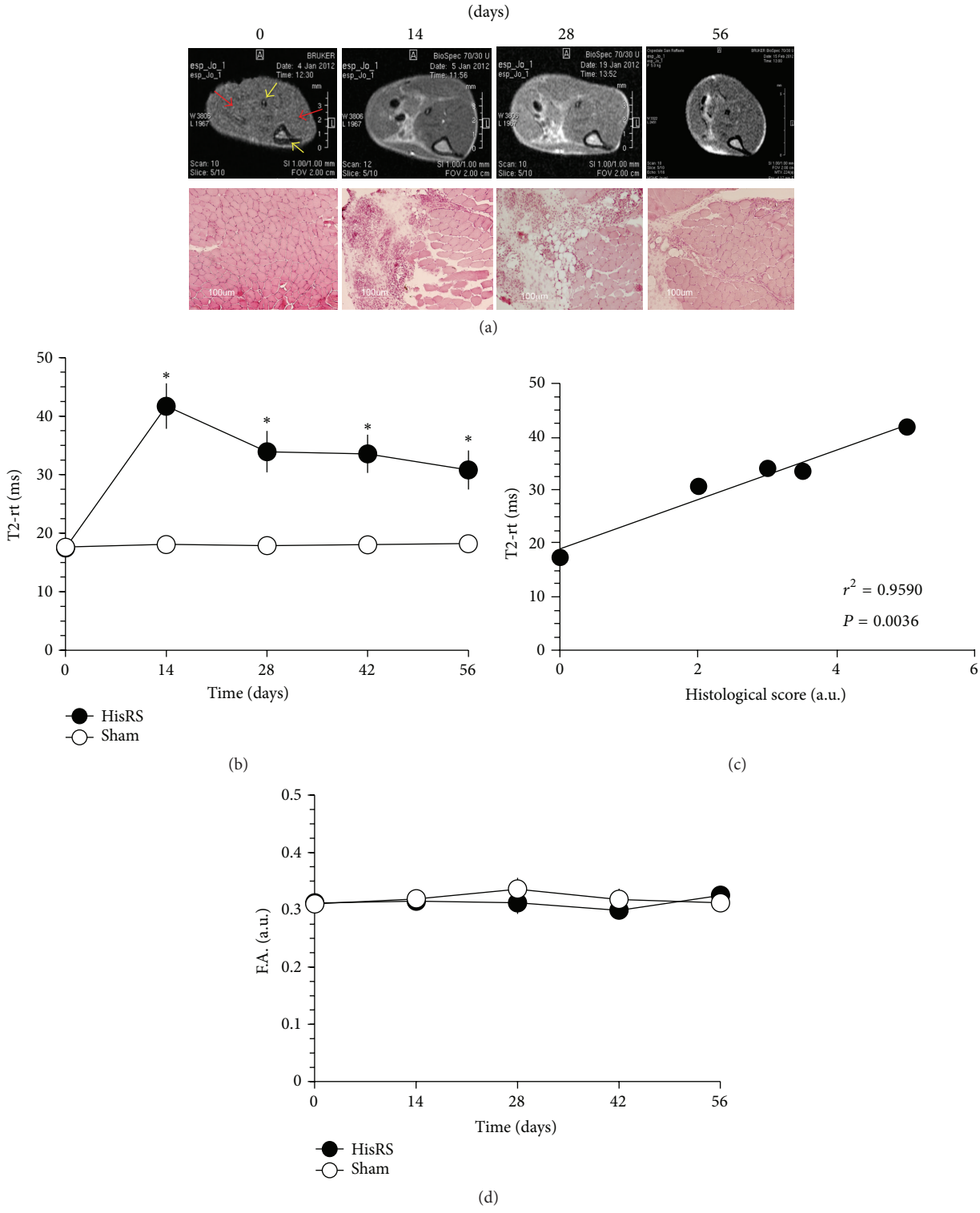


FIGURE 2: *T2 MRI mapping reflects inflammation and remodeling in experimental HisRS-induced myositis*. Representative T2-weighted images of immunized mice ((a) upper panels) obtained before (time 0) and 14, 28, or 56 days after treatment reveal ongoing inflammatory involvement and are well compatible with results obtained at the same time points by histopathology in selected animals ((a) lower panels, H&E). Yellow arrows indicate the fibula (upper) and tibia (lower) bones, while red arrows show skeletal muscles (GS left and TA right). The graphs show the dynamic trends of T2-rt ((b) y-axis) and F.A. ((d) y-axis) in the longitudinal study of immunized and sham-treated mice before and at various times after treatment (x-axis, days). $n \geq 6$; *statistically different from sham-treated mice; $P < 0.05$. The graph in (c) depicts correlation analysis between T2-rt and the histological score that reflects muscle damage and infiltration (see Methods). $n = 3$ for each time point. The time points considered for the correlation analysis were days 0, 14, 28, 42, and 56.

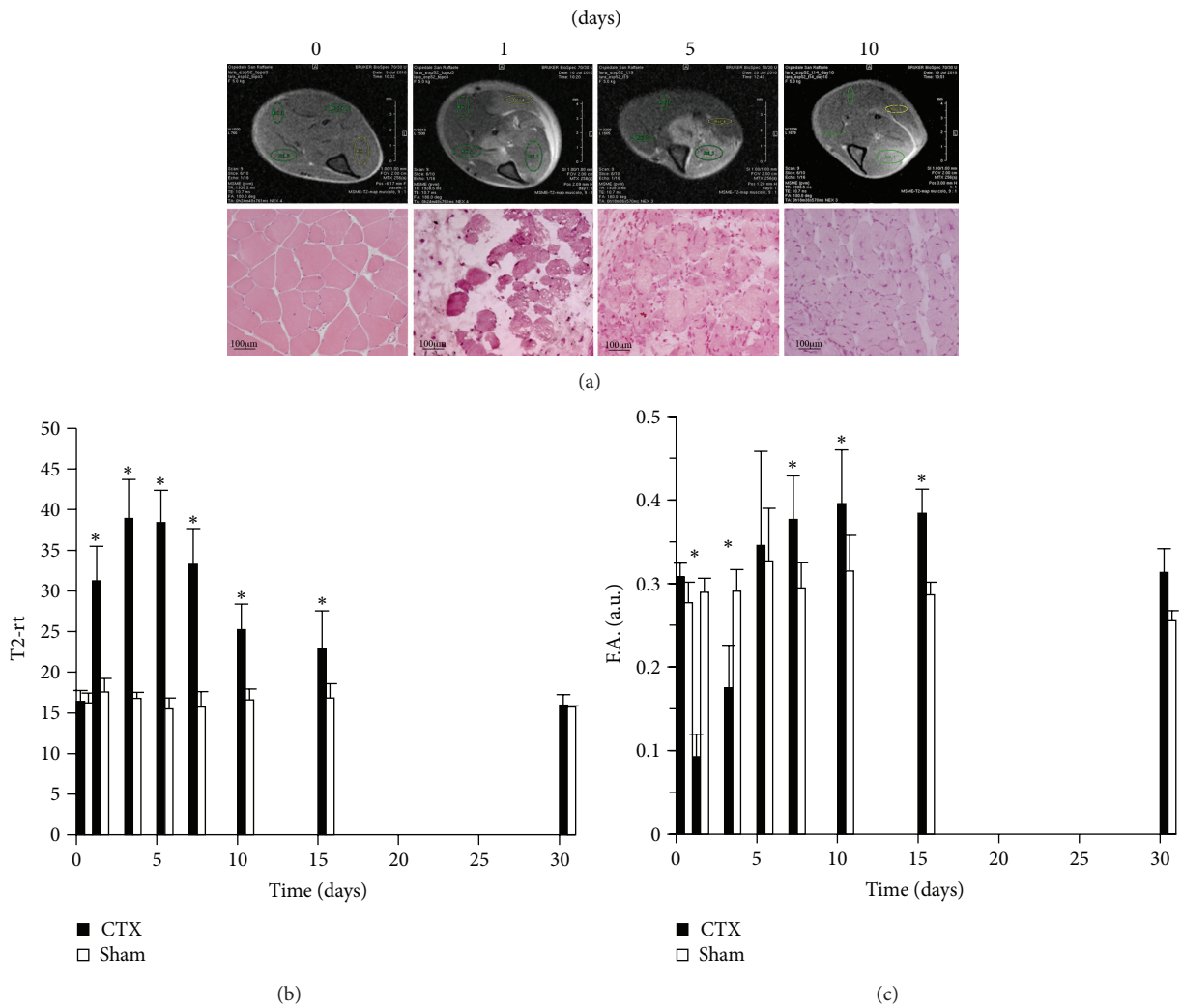


Figure 3: *T2 and DTI MRI mapping reflect independent inflammatory events in acutely injured muscles.* Representative T2-weighted images before (time 0) and at various times (1, 5, or 10 days) after intramuscular CTX injection ((a) upper images). Results are compatible with those obtained at the same time points by histopathology in selected animals ((a) lower panels, H&E). The graphs show the dynamic trends of T2-rt ((b) y-axis) and F.A. ((c) y-axis) in the longitudinal study of damaged (CTX) and sham-treated mice before and at various times after treatment (x-axis, days). $n \geq 6$; *statistically different from sham-treated mice; $P < 0.05$.

has been rather cumbersome, since by definition it requires histopathological studies and animal sacrifice.

While replacement is difficult to achieve in this field of research, given the complexity of events involved in myositis pathogenesis that can be only reproduced in *in vivo* systems, the availability of noninvasive tools to monitor muscle inflammation will permit reductions in the number of animals necessary for experimental studies and increase the amount of relevant information that can be derived from single animals followed over time. At the same time, this imaging modality will complement information derived from histopathological studies that encompass only limited areas of tissue and therefore suffer from potential sampling bias.

Here, we have focused on MRI-based imaging techniques that have been previously shown to represent noninvasive and quantitative tools for the assessment of muscle inflammation and remodeling in experimental animals [13, 16, 21, 24, 25]. We utilized a biologically relevant model of experimental myositis, which can be elicited by immunization of wild-type mice with a synthetic sequence corresponding to a well-characterized autoantigen, HisRS [11, 12]. This model triggers anti-HisRS autoantibodies as well as self-sustaining injury and leukocyte infiltration of skeletal muscle, reproducing key features of spontaneous human disease that can also be associated with fever, arthritis, mechanic's hands, Raynaud's phenomenon, and interstitial lung disease as part of the anti-synthetase syndrome [26]. Reference [27] shows that muscle inflammation has specific features in these patients with the antisynthetase syndrome, with predominant involvement of perimysial connective tissue and less significant peripheral atrophy or microvascular injury [28, 29].

As shown by our results, MRI is a powerful tool to investigate muscle inflammation occurring in the mouse model of HisRS-induced myositis. Most significantly, cohorts

of mice can be longitudinally followed with this technique that accurately detects specific histopathological features of the events taking place in the tissue upon autoimmunity. In our study, MRI proved extremely sensitive, detecting early tissue changes associated with relatively minor increases in the extent of muscle necrosis revealed by changes in serum CPK levels. Importantly, MRI of sham-treated animals remained stable over the 8 weeks of the study, effectively ruling out major effects related to the immunization vehicle, IFA.

Overall, our study provides strong evidence of the link between HisRS-induced immune responses and myositis that occurs, as early as fourteen days after immunization. Moreover, noninvasive T2 mapping reveals the involvement of perimysial connective tissue, that is, a specific feature of the human antisynthetase syndrome [28], clearly differentiating HisRS-induced myositis from the CTX model of acute, self-limited muscle necrosis. As such, the T2-rt values effectively mirror the extent and distribution of muscle inflammation, not only in the setting of homeostatic responses to sterile toxic injury [21], but also in a more complex and long-lasting model of immune-mediated muscle remodeling. Of importance, the degree of anisotropy of water diffusivity remained substantially stable in the mice that we have studied upon myositis induction, contrasting sharply with MRI results obtained in CTX-treated mice. Because this parameter largely reflects the diffusion of water in intracellular spaces [24, 30], these results are in agreement with the preferential involvement of the perimysial tissue (reflected by clear histological evidence of edema in the perifascicular space) and the substantial conservation of tissue architecture in HisRS-induced myositis.

Based on these observations, our findings indicate that MRI represents a versatile tool that can sensitively detect inflammatory processes associated with various types of muscle injury. At the same time, this imaging modality affords the opportunity to noninvasively monitor more subtle modifications in the architecture and inflammatory state of skeletal muscle, including those changes associated with initial age related decline in muscle mass and simultaneous increases of both T2-rt and F.A. Further studies are warranted to verify whether specific MRI codes are also associated with other conditions characterized by persistent inflammation and wasting of the skeletal muscle.

5. Conclusions

In conclusion, our study was designed to characterize the ability of MR imaging to reveal specific inflammatory events in the skeletal muscle of mice with HisRS-induced myositis. Our results provide an in-depth noninvasive characterization of this myositis model, confirming the possibility to link HisRS-induced immune responses and skeletal muscle inflammation to MRI T2-rt changes. This technique allows increasing the amount of relevant information that can be derived from single animals followed over time and may strongly improve and facilitate the study of new therapeutic strategies for human disease as well as of the immunological and local events involved in myositis pathogenesis. At the same time, this imaging modality can help histopathological studies encompassing their potential sampling bias with complement quantitative information. The possibility to follow the same animal from the beginning to the end of treatment and to analyze more parameters at ones, can allow better statistically evaluation of data and underlining the concept that strong efforts should be dedicated to the refinement of experimental procedures that limit the number of animals needed for experimental models of human disease.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This work was supported by the Italian Ministry of Health (Fondo per gli Investimenti della Ricerca di Base-IDEAS to Patrizia Rovere-Querini and Ricerca Finalizzata 2011 to Patrizia Rovere-Querini and Angelo A. Manfredi), by the Associazione Italiana Ricerca sul Cancro (AIRC IG11761 to Angelo A. Manfredi), and by the Italian Ministry of University and Research (PRIN 2010 to Angelo A. Manfredi).

References

[1] S. Rayavarapu, W. Coley, T. B. Kinder, and K. Nagaraju:, "Idiopathic inflammatory myopathies: pathogenic mechanisms of muscle weakness," *Skelet Muscle*, vol. 3, p. 13, 2013.

[2] A. Bender, N. Ernst, A. Iglesias, K. Dornmair, H. Wekerle, and R. Hohlfeld, "T cell receptor repertoire in polymyositis: clonal expansion of autoaggressive CD8+ T cells," *Journal of Experimental Medicine*, vol. 181, no. 5, pp. 1863–1868, 1995.

[3] R. Hohlfeld and A. G. Engel, "Coculture with autologous myotubes of cytotoxic T cells isolated from muscle in inflammatory myopathies," *Annals of Neurology*, vol. 29, no. 5, pp. 498–507, 1991.

[4] J. M. Pandya, A. E. R. Fasth, M. Zong et al., "Expanded T cell receptor Vβ-restricted T cells from patients with sporadic inclusion body myositis are proinflammatory and cytotoxic CD28null T cells," *Arthritis and Rheumatism*, vol. 62, no. 11, pp. 3457–3466, 2010.

[5] J. H. Choi, Y. E. Park, S. I. Kim et al., "Differential Immunohistological features of inflammatory myopathies and dysferlinopathy," *Journal of Korean Medical Science*, vol. 24, no. 6, pp. 1015–1023, 2009.

[6] A. Ghirardello, N. Bassi, L. Palma et al., "Autoantibodies in polymyositis and dermatomyositis," *Current Rheumatology Reports*, vol. 15, p. 335, 2013.

[7] A. L. Mammen, "Autoimmune myopathies: autoantibodies, phenotypes and pathogenesis," *Nature Reviews Neurology*, vol. 7, no. 6, pp. 343–354, 2011.

[8] P. Venalis and I. E. Lundberg, "Immune mechanisms in polymyositis and dermatomyositis and potential targets for therapy," *Rheumatology (Oxford)*, vol. 53, no. 3, pp. 397–405, 2014.

[9] K. B. Stone, C. V. Oddis, N. Fertig et al., "Anti-Jo-1 antibody levels correlate with disease activity in idiopathic inflammatory

myopathy," *Arthritis and Rheumatism*, vol. 56, no. 9, pp. 3125–3131, 2007.

[10] Y. Katsumata and D. P. Ascherman, "Animal models in myositis," *Current Opinion in Rheumatology*, vol. 20, no. 6, pp. 681–685, 2008.

[11] Y. Katsumata, W. M. Ridgway, T. Oriss et al., "Species-specific immune responses generated by histidyl-tRNA synthetase immunization are associated with muscle and lung inflammation," *Journal of Autoimmunity*, vol. 29, no. 2-3, pp. 174–186, 2007.

[12] M. Soejima, E. H. Kang, X. Gu, Y. Katsumata, P. R. Clemens, and D. P. Ascherman, "Role of innate immunity in a murine model of histidyl-transfer RNA synthetase (Jo-1)-mediated myositis," *Arthritis and Rheumatism*, vol. 63, no. 2, pp. 479–487, 2011.

[13] G. P. Kuo and J. A. Carrino, "Skeletal muscle imaging and inflammatory myopathies," *Current Opinion in Rheumatology*, vol. 19, no. 6, pp. 530–535, 2007.

[14] P. O. Carstens and J. Schmidt:, "Diagnosis, pathogenesis and treatment of myositis: recent advances," *Clinical & Experimental Immunology*, vol. 175, no. 3, pp. 349–358, 2014.

[15] R. V. Curiel, R. Jones, and K. Brindle, "Magnetic resonance imaging of the idiopathic inflammatory myopathies: structural and clinical aspects," *Annals of the New York Academy of Sciences*, vol. 1154, pp. 101–114, 2009.

[16] A. B. McMillan, D. Shi, S. J. P. Pratt, and R. M. Lovering, "Diffusion tensor MRI to assess damage in healthy and dystrophic skeletal muscle after lengthening contractions," *Journal of Biomedicine and Biotechnology*, vol. 2011, Article ID 970726, 10 pages, 2011.

[17] L. M. McIntosh, R. E. Baker, and J. E. Anderson, "Magnetic resonance imaging of regenerating and dystrophic mouse muscle," *Biochemistry and Cell Biology*, vol. 76, no. 2-3, pp. 532–541, 1998.

[18] S. J. Pratt, S. Xu, R. J. Mullins, and R. M. Lovering, "Temporal changes in magnetic resonance imaging in the mdx mouse," *BMC Research Notes*, vol. 6, p. 262, 2013.

[19] S. Tardif-de Géry, J. Vilquin, P. Carlier et al., "Muscular transverse relaxation time measurement by magnetic resonance imaging at 4 Tesla in normal and dystrophic dy/dy and dy(2j)/dy(2j) mice," *Neuromuscular Disorders*, vol. 10, no. 7, pp. 507–513, 2000.

[20] S. Schmidt, A. Vieweger, M. Obst et al., "Dysferlin-deficient muscular dystrophy: gadofluorine M suitability at MR imaging in a mouse model," *Radiology*, vol. 250, no. 1, pp. 87–94, 2009.

[21] A. Esposito, L. Campana, A. Palmisano et al., "Magnetic resonance imaging at 7T reveals common events in age-related sarcopenia and in the homeostatic response to muscle sterile injury," *PLoS One*, vol. 8, Article ID e59308, 2013.

[22] L. Casciola-Rosen, K. Nagaraju, P. Plotz et al., "Enhanced autoantigen expression in regenerating muscle cells in idiopathic inflammatory myopathy," *Journal of Experimental Medicine*, vol. 201, no. 4, pp. 591–601, 2005.

[23] D. P. Ascherman, "Animal models of inflammatory myopathy," *Current Rheumatology Reports*, vol. 14, no. 3, pp. 257–263, 2012.

[24] B. M. Damon, Z. Ding, A. W. Anderson, A. S. Freyer, and J. C. Gore, "Validation of diffusion tensor MRI-based muscle fiber tracking," *Magnetic Resonance in Medicine*, vol. 48, no. 1, pp. 97–104, 2002.

[25] E. Amarteifio, A. M. Nagel, H. U. Kauczor, and M. A. Weber:, "Functional imaging in muscular diseases," *Insights Imaging*, vol. 2, no. 5, pp. 609–619, 2011.

[26] I. N. Targoff, "Autoantibodies and their significance in myositis," *Current Rheumatology Reports*, vol. 10, no. 4, pp. 333–340, 2008.

[27] L. Casciola-Rosen, "Histidyl-transfer RNA synthetase: a key participant in idiopathic inflammatory myopathies," *Arthritis and Rheumatism*, vol. 63, no. 2, pp. 331–333, 2011.

[28] T. Mozaffar and A. Pestronk, "Myopathy with anti-Jo-1 antibodies: pathology in perimysium and neighbouring muscle fibres," *Journal of Neurology Neurosurgery and Psychiatry*, vol. 68, no. 4, pp. 472–478, 2000.

[29] W. Stenzel, H. H. Goebel, and E. Aronica, "Review: immune-mediated necrotizing myopathies—a heterogeneous group of diseases with specific myopathological features," *Neuropathology and Applied Neurobiology*, vol. 38, no. 7, pp. 632–646, 2012.

[30] B. M. Damon, D. M. Wigmore, Z. Ding, J. C. Gore, and J. A. Kent-Braun, "Cluster analysis of muscle functional MRI data," *Journal of Applied Physiology*, vol. 95, no. 3, pp. 1287–1296, 2003.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 965631, 11 pages
http://dx.doi.org/10.1155/2014/965631

Review Article

Understanding the Process of Fibrosis in Duchenne Muscular Dystrophy

Yacine Kharraz, Joana Guerra, Patrizia Pessina, Antonio L. Serrano, and Pura Muñoz-Cánoves

Cell Biology Group, Department of Experimental and Health Sciences, Pompeu Fabra University (UPF), CIBER on Neurodegenerative diseases (CIBERNED), Institució Catalana de Recerca i Estudis Avançats (ICREA), Doctor Aiguader 83, 08003 Barcelona, Spain

Correspondence should be addressed to Antonio L. Serrano; antonio.serrano@upf.edu
and Pura Muñoz-Cánoves; pura.munoz@upf.edu

Received 5 March 2014; Accepted 8 April 2014; Published 4 May 2014

Academic Editor: Marina Bouché

Fibrosis is the aberrant deposition of extracellular matrix (ECM) components during tissue healing leading to loss of its architecture and function. Fibrotic diseases are often associated with chronic pathologies and occur in a large variety of vital organs and tissues, including skeletal muscle. In human muscle, fibrosis is most readily associated with the severe muscle wasting disorder Duchenne muscular dystrophy (DMD), caused by loss of dystrophin gene function. In DMD, skeletal muscle degenerates and is infiltrated by inflammatory cells and the functions of the muscle stem cells (satellite cells) become impeded and fibrogenic cells hyperproliferate and are overactivated, leading to the substitution of skeletal muscle with nonfunctional fibrotic tissue. Here, we review new developments in our understanding of the mechanisms leading to fibrosis in DMD and several recent advances towards reverting it, as potential treatments to attenuate disease progression.

1. Introduction

Duchenne muscular dystrophy (DMD) is a fatal, X-linked recessive disorder characterized by a progressive loss of muscle mass and function [1]. DMD has a prevalence of approximately 1 in 3,500 live male births and is caused by mutations in the dystrophin gene that precludes the synthesis of a full-length and/or fully functional protein. Dystrophin itself is a large structural protein that stabilizes the sarcolemma of muscle fibers. In its absence, fibers become vulnerable to contraction-induced damage and undergo cycles of necrosis and repair until the muscle mass becomes replaced by fat and fibrous tissue. Affected boys become confined to a wheelchair and normally live until the late teens or early twenties. There is currently no cure and patients only receive palliative care. Consequently, there has been a considerable and sustained effort to uncover the mechanisms of disease and develop new treatment possibilities [2, 3]. Attempts to provide a primary treatment of DMD include viral replacement therapy, plasmid-mediated nonviral expression, stem cell transplantation, antisense oligonucleotide-induced exon skipping, and nonsense mutation suppression by drugs, amongst others, although they remain unsuccessful [4–7].

Recently, however, attention has also begun to focus on understanding and modifying the pathological background of the disease, it is now well established that many of the pathological features of DMD are not only caused by the lack of dystrophin and/or the failure of muscle stem cells (also called satellite cells) but are also due to the complex interactions of these cells with the surrounding environment. Changes in this environment can delay muscle repair and regeneration and enhance inflammation, leading to disease exacerbation and fibrosis development. Important contributors to DMD pathogenesis, and potential obstacles or targets for achieving better therapeutic outcomes, include the inflammatory components of the damaged and regenerating muscle and the auxiliary cell mediators such as fibroblasts that support satellite and inflammatory cells, as well as the milieu of soluble factors produced by them. In particular, recent studies have highlighted the importance of all these

cells and factors in the development of not only fibrosis arising during aberrant regeneration and DMD progression, but in other diseases such as diabetes and in aging (reviewed in [8]).

Fibrosis is defined as the excessive or unregulated deposition of extracellular matrix (ECM) components and is a particular hallmark of DMD and abnormal repair processes in several other tissues upon injury including liver, lung, kidney, and pancreas. Controlled deposition of ECM components during growth and repair is critical for providing a scaffold to build and structure new tissue, but alterations in the timing, the intensity, and/or the components of this process can lead to excessive ECM deposition (fibrosis) and loss of tissue function. Fibrosis has a double negative consequence for the potential treatment of DMD in that it not only alters muscle function, but also reduces the amount of target muscle available for therapy and repair. Therefore, a better understanding of the components and processes leading to the development of fibrosis is important for our ability to improve muscle repair, treat DMD, and potentially restore muscle function. Although this review focuses on DMD, there is evidence from other myopathies that a dysregulated and/or disordered ECM may also contribute to disease progression [9, 10].

A review of all of the contributing factors that lead to fibrosis is beyond the scope of this report, although several recent reviews are available [11, 12]. Instead we focus here on some of the recent developments that reveal new or unexpected roles of some of these cell types and molecular effectors of skeletal muscle fibrosis, giving particular emphasis on fibrosis in DMD patients, as well as in animal models such as the commonly used dystrophic mdx mouse. In particular we highlight some new developments in the understanding of the TGFβ signaling pathway, perhaps the most critical effector of fibrosis, and in particular its interaction with connective tissue growth factor (CTGF) and the renin angiotensin system (RAS). We also review recent efforts to lineage trace cellular sources of ECM production and assess the novel fibrogenic role assigned to other nonsatellite cell types that have been identified in muscle. Finally, we will also describe some of the recent progress in the development and characterization of animal models for the study of fibrosis *in vivo* and some potential therapeutic approaches to combat and diminish fibrosis in DMD.

2. Growth Factors in Fibrosis Development

2.1. The TGFβ Signaling Pathway. One of the most potent profibrogenic factors described *in vivo* is transforming growth factor-beta (TGFβ) as reviewed in [13]. TGFβ is initially generated as a latent precursor of one of three isoforms TGFβ1, TGFβ2, and TGFβ3 [14]. Latent TGFβ is stored in the ECM where it is activated upon tissue damage or specific growth signals (reviewed in [15]). Activated TGFβ binds to a heterodimeric complex consisting of one TGFβ type I receptor molecule, also called activin linked kinase 5 (ALK5), and one TGFβ type II receptor. Importantly, TGFβ is expressed in regenerating muscle after injury, as well as in the dystrophic muscle of DMD patients and mdx mice [14, 16, 17], where it stimulates fibroblasts to produce ECM proteins like collagen and fibronectin. In addition, TGFβ and other profibrogenic polypeptides can be produced by infiltrating immune, inflammatory, mesenchymal, and tissue-specific cells (reviewed in [18]).

When TGFβ is liberated it can signal via the canonical TGFβ pathway (see below) or through several alternative pathways (Figure 1). Importantly, changes in the level of signal transduction via these different pathways have been shown to modulate fibrotic effects and therefore their various signaling mediators are potential targets for antifibrotic therapies. In normal fibroblasts the canonical pathway passes through ALK5 which phosphorylates transcription factors Smad2 and 3. These signal transducers then bind to Smad4 to form a complex that is translocated to the nucleus to activate transcription of profibrotic genes (reviewed in [19, 20]). Alternatively, TGFβ may also signal via additional intracellular transducers such as the Ras/MEK/ERK pathway, the p38 MAPK pathway, the c-abl pathway, and JNK as additional intracellular mediators [21]. Signaling via these alternative pathways is able to modify gene expression in a promoter-selective fashion. ERK, for example, is normally required for collagen type I expression, whereas other signaling molecules like FAK, JNK, and TAK1 are required for divergent processes such as ECM contraction and myofibroblast differentiation [22].

Recently, several indirect interactions between TGFβ signaling and other pathways have been reported. For instance, decreased insulin-like growth factor (IGF) signaling in IGF-1R(+/−) heterozygous mice deleted for the IGF-1 receptor in skeletal muscle using a muscle specific MyoD-Cre driver resulting in impaired regeneration, depressed expression of MyoD and myogenin, and increased expression of TGFβ1, α-SMA, and collagen I and fibrosis [23]. Further mechanistic studies showed that in myoblasts, IGF-1 treatment could inhibit TGFβ1-stimulated Smad3 phosphorylation and increase phosphorylated-AKT- (P-AKT-) Smad3 interactions, thus impeding nuclear translocation of Smad3 and thereby reducing the expression of fibrotic genes. Conversely, a reduced amount of IGF-1R diminished the levels of P-AKT, allowing dissociation and nuclear translocation of Smad3 and enhancement of the TGFβ1 signaling pathway and fibrosis [23].

TGFβ can also decrease the production of enzymes that degrade the ECM, while simultaneously increasing production of proteins like tissue inhibitors of metalloproteinases (TIMPs) and plasminogen activator inhibitor type-1 (PAI-1, see below) that inhibit ECM-degrading enzymes. Consistent with this, injection of recombinant TGFβ into skeletal muscle *in vivo* has two effects. Firstly, it stimulates TGFβ expression in myogenic cells (among other cells) in an autocrine fashion and secondly, it induces connective tissue production in the area of the injection (reviewed in [24]). The same study has shown that C2C12 myogenic cells overexpressing TGFβ can differentiate into myofibroblastic cells after intramuscular transplantation [25]. This process can be inhibited by the small leucine-rich proteoglycan decorin, which binds to and inhibits TGFβ [26]. Similar studies in transgenic mice overexpressing TGFβ1 in a muscle-specific manner showed

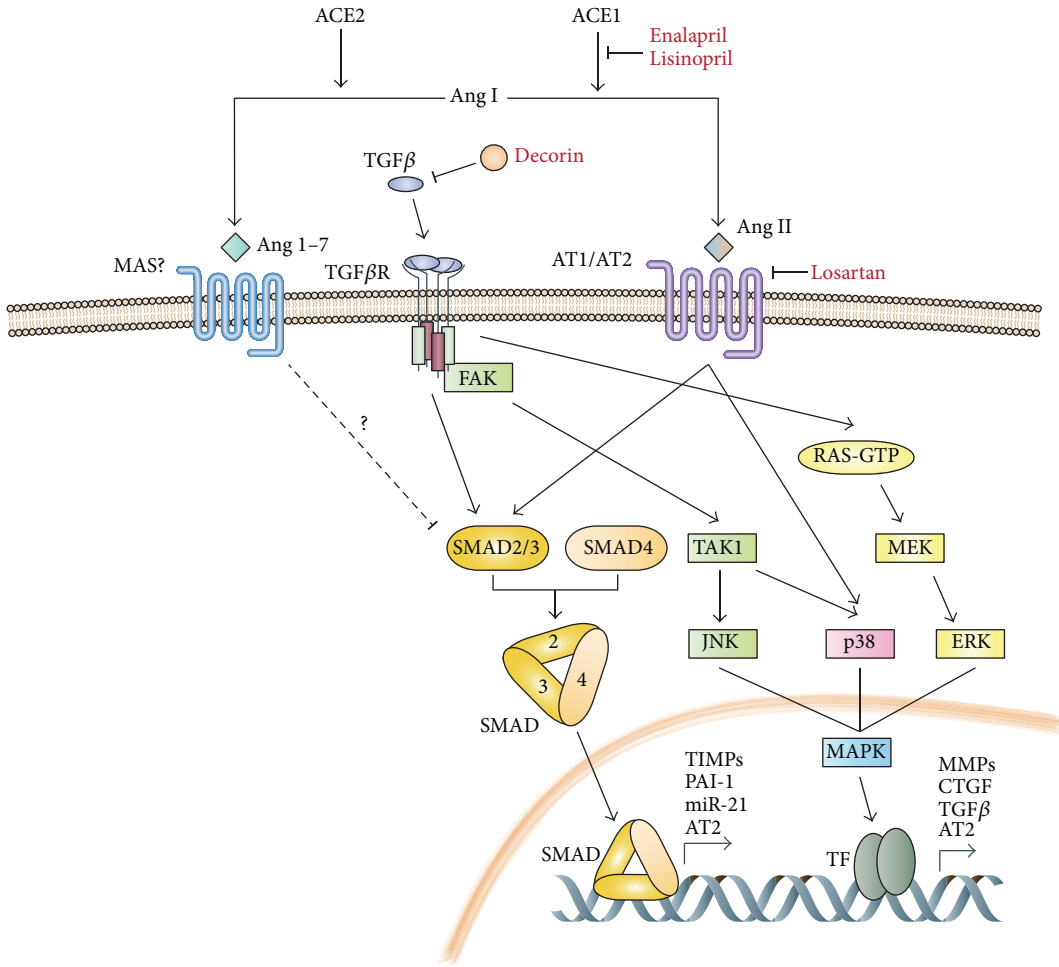


Figure 1: Crosstalk between TGFβ signaling and the renin-angiotensin system in fibrosis. TGFβ can signal via its canonical pathway, involving Smad proteins, or through several alternative pathways such as the p38 MAPK signaling or the RAS/ERK MAPK signaling pathways. Both canonical and alternative pathways lead to expression of molecules implicated in fibrosis such as CTGF or PAI-1. Similarly, Ang II signals through AT1 or AT2 and can also activate Smad proteins and the p38 MAPK signaling pathway, leading to increased expression of profibrotic genes. Ang 1–7 has an opposite effect, inhibiting the canonical TGFβ pathway. Antifibrotic molecules inhibiting RAS or the TGFβ signaling are indicated in red.

muscle wasting and endomysial fibrosis [27]. Finally, latent TGFβ-binding protein 4 (LTBP4), which regulates the release and bioavailability of TGFβ from the ECM, was recently shown to modulate fibrosis in mdx mice although its role in human DMD, if any, has not yet been established [28].

2.2. Crosstalk between TGFβ and Other Growth Factors: CTGF and the Renin-Angiotensin System. Connective tissue growth factor (CTGF) is a nonstructural regulatory protein present in the ECM that has an important role in fibrosis. Skeletal muscle from DMD patients, dystrophic dogs, and mdx mice all show elevated levels of CTGF [29]. Fibrosis development can be reduced in mdx mice by systemic administration of a neutralizing antibody against CTGF [30]. Functionally, CTGF has the ability to reproduce or amplify the effects of TGFβ on fibrosis. For example, it can induce collagen type 1, α5 integrin, and fibronectin much more potently than TGFβ in fibroblasts [31]. Moreover, overexpression of CTGF in muscle of WT mice with an adenovirus vector carrying the CTGF cDNA sequence induces strong fibrosis [32]. Like for TGFβ, decorin may also negatively regulate CTGF activity. Even if the exact mechanism is not known, it has been shown that the inhibitory action of decorin on CTGF activity depends on its capacity to directly bind to CTGF [33]. Interestingly, the same study showed that CTGF induces the expression of decorin, indicating a potential mechanism of autoregulation. Together, these results suggest a negative role for both TGFβ and CTGF in muscular dystrophies by directly inducing fibrotic processes and inhibiting myogenesis [34]. A possible new role for CTGF in fibrosis was recently revealed by the observation that CTGF expression is decreased in dystrophic muscle when angiotensin-converting enzyme (ACE) is inhibited by enalapril [35]. ACE is a critical enzyme of the renin-angiotensin system (RAS) that regulates blood pressure. Angiotensinogen is mainly produced by

the liver and then converted into angiotensin 1 (Ang 1) by the renin enzyme (also known as angiotensinogenase), which is secreted by the granular cells of the kidney. Ang 1 is subsequently converted into angiotensin 2 (Ang 2) by ACE (Figure 1). Angiotensin 2 produces its biological effect by signaling through the Ang 2 receptor type 1 (AT1) and the Ang 2 receptor type 2 (AT2). Over the past decade, various studies have showed that Ang 2 is involved in the development of fibrosis in different pathogenic conditions and organs [36]. Interestingly, it was shown that the RAS is activated in various muscular dystrophies such as DMD or congenital muscular dystrophy (CMD) [37] and that dystrophic muscle in humans has increased levels of ACE, which may explain the elevated CTGF levels, but this remains to be proved. Although the role of the RAS system in fibrosis is not completely clear, several studies have shown that Ang 2 also induces expression of fibrotic markers in myoblasts [38]. TGFβ treatment of C2C12 myoblasts was also shown recently to significantly increase AT2 expression [39], therefore providing a putative link between TGFβ signaling and CTGF expression. Interestingly, while Ang 2 seems to be profibrotic, recent data indicates that angiotensin 1–7 (Ang 1–7), an endogenous bioactive peptide derived from Ang 2, has opposite effect to Ang 2. Indeed, it has been shown that Ang 1–7 inhibits TGFβ-Smad signaling *in vivo*, which in turn leads to the reduction of the profibrotic microRNA- (miR-) 21, decreasing greatly fibrosis development in dystrophic muscles of mdx mice [40].

3. ECM Remodeling by Matrix Proteases

In addition to molecules like TGFβ and CTGF that promote ECM deposition, normal muscle repair also requires factors that regulate the proteolytic degradation of the ECM, for example, to facilitate satellite cell and myoblast migration, to remove the temporary scaffold laid down during regeneration, and to allow fiber growth. Clearly, if these factors become dysregulated, then ECM deposition, fibrosis, and loss of muscle function could ensue. We will only consider here recent developments to this group of molecules, which include serine proteases of the plasminogen activation (PA) system, the broad family of matrix metalloproteinases (MMPs), and their inhibitors, PAI-1 and the TIMPs, respectively (reviewed in [41, 42]). Several other publications cover more general aspects of their role in skeletal muscle and fibrosis [43]. The MMPs are a large family of zinc-dependent proteolytic enzymes that includes various collagenases (MMP-1, MMP-8, and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-7, MMP-10, and MMP-11), membrane-type metalloproteinases (MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, and MMP-25), and metalloelastase (MMP-12) [41]. MMPs facilitate inflammation and migration of myogenic, inflammatory, vascular, and fibroblastic cells to damaged tissue by degrading the ECM. MMPs are released from damaged muscle and infiltrating cells, but their function is controlled not only by simple expression and release, but also by the net MMP activity which reflects the relative amount of activated enzyme. MMP activation requires proteolytic cleavage of the inactive precursor, by either membrane-type matrix metalloproteinase 1 (MT1-MMP) [44] or plasmin, and cleavage of their corresponding inhibitors [45].

Regulation of MMPs is thus complicated and involves not only expression, but also activation. In addition, in some cases the activity of MMPs can amplify or synergize with serine proteases of the plasminogen activation system to mediate ECM remodeling during tissue repair. The main function of the PA system is to degrade fibrin and at its core is the zymogen plasminogen which is converted into the active enzyme, plasmin, by two plasminogen activators (PAs): tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). There are several inhibitors of the PA system including plasminogen activator inhibitor 1 (PAI-1) and alpha2-antiplasmin that operate at the level of the PAs or plasmin, respectively [42]. The PA system plays an important role in muscular dystrophy. Studies in mdx mice have detected increased uPA expression in skeletal muscle, while genetic loss of uPA exacerbated dystrophy and impaired muscle function in mdx mice because uPA is required to prevent excessive fibrin deposition [46]. More recently it has been shown that the extracellular PAI-1/uPA balance is an important regulator of miR-21 biogenesis, controlling age-associated muscle fibrosis and disease severity in muscle dystrophy [17]. Genetic loss of PAI-1 in mdx mice was shown to promote muscle fibrosis through several mechanisms. Firstly, it altered collagen metabolism by increasing uPA-mediated proteolytic processing of TGFβ in muscle fibroblasts. Secondly, it also activated miR-21 expression, which in turn inhibited PTEN and enhanced signaling via the AKT pathway, which controls cell proliferation and survival, thus endowing TGFβ with a remarkable ability to promote cell proliferation. However, age-associated fibrogenesis and muscle deterioration in mdx mice, as well as advanced muscle fibrosis in young mdx mice lacking PAI-1, could be prevented by direct interference with miR-21 or the PAI-1 substrate uPA. Consistent with this, forced miR-21 overexpression in mdx muscle accelerated fibrosis and enhanced disease severity, whereas treatment of aged mdx mice with an inhibitor of miR-21 improved muscle homeostasis and reduced fibrosis. This is an important observation since fibrosis is normally considered irreversible at advanced ages. Further studies are needed to refine our understanding of the role of the PAI-1/miR-21 fibrogenic axis in skeletal muscle fibrosis and the disease course in DMD patients, but as the full role of the axis emerges, so too will new therapeutic targets. From all these findings, it seems that the pronounced fibrosis in human dystrophic muscles is at least partially related to an altered proteolytic activity in the dystrophic muscles due to imbalances in expression and activity of PA/MMP system components [47].

4. Inflammation-Driven Fibrosis

One of the hallmarks of DMD is the chronic cycles of myofiber necrosis and repair, which histologically manifest as a sustained infiltration of mononuclear cells in muscle tissue.

Consequently, considerable efforts have gone into characterizing these inflammatory cells to define their functions and their differences between the various acute and chronic (transgenic/genetic) models of skeletal muscle injury. One of the first events after tissue damage is the invasion of inflammatory cells to the site of injury. In response to acute injury, the first cells infiltrating the muscle are mainly leukocytes belonging to the myeloid lineage, principally neutrophils and monocytes/macrophages ([48], reviewed in [49]). Any disruption in the coordinated initiation, progression, and resolution of inflammation can lead to persistent muscle damage and impairment of regeneration, which in many cases is also characterized by development of fibrosis as observed in the muscular dystrophies (reviewed in [18]).

Indeed, it is well known that fibrosis is preceded and influenced by inflammation in several pathologies. In the context of muscular dystrophy, previous studies showed that inflammatory macrophages are an important source of TGFβ in the mdx diaphragm muscle, suggesting that they might contribute importantly to fibrosis development [14]. However, macrophage-depletion experiments or investigation of mouse models with impaired macrophage recruitment demonstrated a more complex role for macrophages in muscle repair and the mdx phenotype, being either deleterious [50] or beneficial for fibrosis development [46], depending on the model used for the study. Increasing evidence supports a key role of M2 "alternatively activated" macrophages in the development of fibrotic conditions, such as asthma and idiopathic lung fibrosis, as opposed to M1 "classically activated" proinflammatory macrophages [51]. These M2 macrophages are activated by Th2-derived cytokines, such as IL-13, and can be identified by specific cell surface markers such as CD206 (also known as mannose receptor). They also express high levels of TIMP-1 and the chitinase-like secretory lectins Ym1 and Ym2. Interestingly, TGFβ, in conjunction with IL-13, may amplify the expression of arginase I in alternatively activated macrophages which is a key enzyme in the initiation of collagen synthesis by fibroblasts. Indeed arginase I produces the amino acid proline that is required for collagen synthesis [52]. Of note, the diaphragm of mdx mice contains CD206 positive macrophages expressing arginase I and TGFβ, whose expression increases with age correlating with increased levels of IL-13 [16] suggesting the potential implication of Th2/alternative macrophage activation in dystrophic muscle fibrosis. Interestingly, muscles of DMD patients also show a correlation between the number of alternatively activated macrophages and collagen deposition [53]. Other studies have confirmed the presence of a subpopulation of alternatively activated macrophages, the M2c macrophages, a type of alternatively activated macrophage induced by the anti-inflammatory cytokine IL-10 and that express arginase, along with classically activated M1 macrophages within the dystrophic muscle of mdx mice [54]. Indeed, it has been shown that arginine metabolism by alternatively activated macrophages promotes cardiac and muscle fibrosis in mdx muscular dystrophy [55]. However, further investigations are required to elucidate the precise role of alternatively activated macrophages in fibrosis development in muscular dystrophies.

Although M1 macrophages are important for satellite cell proliferation (reviewed in [49]), they could potentially be profibrotic in pathogenic conditions by sustaining chronic inflammation. This is illustrated by the proinflammatory role of fibrinogen in muscular dystrophies. Fibrinogen accumulates in the dystrophic muscles of mdx mice and DMD patients [16, 46] and its genetic and pharmacological depletion in mdx mice can greatly reduce fibrosis development in the diaphragm [16]. Interestingly, fibrinogen modulates inflammation by signaling through the $\alpha_M\beta_2$ integrin (Mac-1), which is expressed on myeloid cells, and induces expression of proinflammatory cytokines and chemokines ([16] and reviewed in [56]), thereby potentially promoting muscle degeneration. Indeed, mice expressing a fibrinogen molecule mutated in its $\alpha_M\beta_2$-binding motif [16, 57] develop less fibrosis in the diaphragm concomitantly with a decreased infiltration of macrophages and decreased expression of proinflammatory cytokines, correlating with improved muscle regeneration.

Along with myeloid cells, lymphocytes have also been shown to play a role in muscular dystrophies. Among this family, a potential role of T helper (Th) and cytotoxic T cells (CTL) in fibrosis development has been raised recently. One of the most interesting animal models, to at least partially reveal the role of these cells in regeneration and fibrosis, is the scid/mdx mouse model. These mice are deficient in functional lymphocytes (both B and T cells), allowing the study of the function of these cells in the progression of the disease. Interestingly, scid/mdx mice develop less fibrosis in the diaphragm at one year of age, which correlates with a decrease in TGFβ protein in the dystrophic muscle [58]. In addition, evidences for the pathogenic role of T cells in the progression of muscular dystrophies come from a study using another mouse model of immunodeficiency, the nu/nu/mdx mice, which lack only functional T cells, but not B cells [59], and showing that T cells contribute to fibrosis progression. Although these models support T cell function in muscle repair and muscular dystrophy, they unfortunately do not discriminate between the pathological effect of CTL and Th cells since both subpopulations are absent in scid/mdx and nu/nu/mdx mice. Using depleting antibodies against either the CD4 antigen (depletion of Th) or the CD8 antigen (depletion of CTL), it was shown that both cell types contribute to aggravation of the pathology in the context of mdx mice [60]. When analyzing the populations of leukocytes infiltrating the dystrophic muscle of mdx mice, Vetrone et al. characterized a subpopulation of T helper cells harboring a Vβ8.1/8.2 TCR (T-cell receptor) and expressing high levels of the ECM protein osteopontin ([61] and reviewed in [62]). Using mdx mice deficient in osteopontin, the authors showed that these mice have less fibrosis in the diaphragm muscle correlating with reduced infiltration of NKT cells and neutrophils. These data suggest that Th cells could mediate their pathogenic effects by controlling migration and/or survival of these inflammatory populations through the secretion of osteopontin.

Th cells can differentiate into different functional types, each of them producing a different profile of cytokines. Their role in fibrosis development in tissues other than skeletal

muscle has been reviewed elsewhere [52, 63]. Unfortunately, since scid/mdx, nu/nu/mdx mice, or CD4 depleted mdx mice are totally deficient in Th cells, these models are unable to reveal the roles of their different polarizations (Th1, Th2, Th17, and Tregs) which probably play nonredundant and even opposite roles in the progression of fibrosis [64].

5. Fibroadipogenic Progenitors in Fibrosis

The existence of progenitor cells sharing characteristics of mesenchymal stem cells (MSCs) in skeletal muscle has been recently discovered [65, 66]. These cells were first named FAPs for fibro/adipogenic progenitors because of their capacity to enter adipose and fibroblast differentiation *in vitro* and *in vivo* [65], and were characterized as nonhematopoietic (CD45_), nonendothelial (CD31_), and nonsatellite cells (α7 integrin- or SM/C2.6- depending on the study), but they did express markers of progenitor cells such as CD34 or Sca-1 [65] or the fibroblast marker PDGFRα [66]. Although it is not assured that these two studies described exactly the same population of cells, it is likely that they are at least closely related or that they overlap. Controversies about their function in myogenesis and normal muscle regeneration exist [67, 68] and have been recently reviewed and discussed elsewhere [69], but it appears that these cells may have a pathogenic role in muscular dystrophies. Indeed, Uezumi et al. showed that fibrosis originates almost exclusively from PDGFRα+ progenitors in the dystrophic muscle of mdx mice [70].

Interestingly, a recent study characterized a subpopulation of cells expressing PDGFRα+, Sca1+, and gp38+, which also transiently expresses Adam12 upon acute injury [71]. Using a lineage tracing system, Adam12 expressing cells were shown to readily differentiate into myofibroblast *in vitro* and *in vivo*. These results suggest that the Adam12+ fraction might be a subpopulation of the FAPS described by Joe et al., which would be more committed to become bona fide fibroblasts. Indeed, depletion of the Adam12+ fraction of MSCs reduced the ECM accumulation induced by cardiotoxin injection. Although this study was performed in an acute model of injury, these findings are consistent with the fact that overexpression of Adam12 in mdx mice aggravates fibrosis [72] and suggests that this population of cells might be a major factor in fibrosis development within dystrophic muscle.

Unlike organs such as kidney or heart, where distinct cell types have been shown to contribute to fibrosis [73, 74], in skeletal muscle very little is known about the role of other cell lineages in fibrosis development and their potential contribution to the pool of fibroblasts. However, although MSCs are probably the main source of fibroblasts within the dystrophic muscle, additional studies suggest that other cells might also contribute to fibrosis in pathological contexts. For example, in aged mice, satellite cells tend to convert from a myogenic to a fibrogenic lineage in response to environmental cues, particularly in response to Wnt signaling [75]. More recently, Zordan and colleagues showed that macrophage infiltration after acute injury is required to sustain the proper differentiation of endothelial-derived progenitors. Indeed, depletion of macrophages after injury led to a transition of endothelial to mesenchymal cells [76].

6. Experimental Mouse Models of Skeletal Muscle Fibrosis

As it becomes increasingly accepted that fibrosis is a crucial component in the pathogenesis of DMD, the need for appropriate mouse models that reflect the human disease has become more and more urgent, since disease progression is less severe in mdx mice than in human patients. In the mdx mouse, fibrosis develops extensively and exclusively in the diaphragm muscle during adulthood [77], while in the more accessible limb muscles, it requires nearly two years for fibrosis to develop and it never reaches the severity of human disease [17]. This is despite limb muscles showing other histological features of the human disease, such as inflammatory infiltration, central nucleation, and both hypertrophied and small calibre fibers. Moreover, in the mdx mouse, several other clinical manifestations are mild in comparison to the human disease [78].

In order to exacerbate or hasten fibrosis, different genetic mouse models have been generated. Utrophin is a large sarcolemmal protein with many structural and functional features similar to dystrophin that is upregulated in muscle of DMD patients and may be able to partially compensate functionally for the loss of dystrophin. Transgenic mdx/utrn$^{+/-}$ mice (mdx mice with haploinsufficiency of utrophin) were generated and were found to have increased inflammation of the hindlimb muscles at 3 and 6 months and in the diaphragm at 3 months only [79]. However, fibrosis was strong in the diaphragm at 6 months, but only mild in hindlimb muscle. Wishing to explore the question of whether differences in muscle stem cell (satellite cell) potential between mice and humans were responsible for the progressive DMD phenotype, Blau and collaborators generated dystrophic mice lacking telomerase activity by crossing C57Bl6 mdx mice with C57Bl6 mice heterozygous for the telomerase RNA component Terc (mTR) [80]. In addition to an enhanced fibrotic phenotype, these mice also had several other phenotypic characteristics of human DMD including profound loss of muscle force, endurance (performance on a treadmill), increased serum creatine kinase (CK) levels, accumulation of calcium deposits within the muscle tissues, and a shortened lifespan [81]. Another study explored differences between mice and humans by focusing on sialic acid composition of glycoproteins and glycolipids. Humans have an inactivating deletion in the CMAH (cytidine monophosphate-sialic acid hydroxylase) gene, which prevents biosynthesis of the sialic acid, N-glycolylneuraminic acid, from all human cells, although in mice this ability is not lost, and just as importantly, glycosylation has been shown to be a modifier of many disease states including mdx mice and α-sarcoglycan-deficient mice [82, 83]. Indeed, genetically engineered CMAH/mdx mice showed increased fibrosis, necrotic foci, and more central nucleation than mdx mice at 6 weeks of age, whereas by 8 months of age mice

had lost ambulation and showed reduced force production. Studies by Ardite and colleages [17] showed that genetic loss of the uPA inhibitor PAI-1 in mdx dystrophic mice enhanced muscle fibrosis via several different mechanisms: firstly, it altered collagen metabolism by uPA-mediated proteolytic processing of TGFβ in muscle fibroblasts; loss of PAI-1 also activated miR-21 expression (through a nongenomic TGFβ-induced Smad activity), which in turn inhibited PTEN (phosphatase and tensin homologue) and enhanced AKT signaling, thereby endowing TGFβ with a remarkable cell proliferation-promoting potential on fibroblasts.

Consistent with the notion that arginine metabolism by arginase-2 in M2 macrophages can drive fibrosis, and that the numbers of arginase-2-expressing M2 macrophages are elevated in the muscle and hearts of dystrophic mice, ablation of arginase-2 in mdx mice resulted in significantly less fibrosis in limb and diaphragm muscles [54]. Conversely, supplementation of young mdx mice with L-arginine promoted a more severe muscle fibrosis than mdx mice treated with D-arginine confirming a role for arginase in fibrosis and disease pathogenesis [54]. Of note, L-Arginine, but not D-arginine, is the natural substrate for nitric oxide synthase (NOS). Thus, caution needs to be taken since many DMD patients are often given arginine supplements, and more studies need to be undertaken to analyze its impact on disease.

A study by Fukada et al. showed that mice in the DBA/2 background exhibit a poor regeneration process after repeated injury. Furthermore, mdx mice in the DBA/2 background (D2-mdx) show severe loss of skeletal muscle weight and higher muscle weakness, while fibrosis and fat accumulation are greatly increased in comparison with mdx mice in the C57Bl10 background [84].

Nevertheless, neither of these mdxmouse models mimics precisely the pathophysiology of human dystrophic disease, and this is one of the reasons why it is difficult to study therapies to stop the progression of fibrosis in DMD.

In addition to genetic models, the drive to overcome our poor understanding of the molecular mechanisms underlying fibrogenesis in DMD has prompted the development of new experimental procedures that can be used to efficiently boost or advance fibrosis in young mdx mice in both hindlimbs without having to wait for its natural physiological onset.

Mechanical muscle injury by daily repeated micromultipunctures for 14 days has been demonstrated to trigger fibrotic lesions in mdx hindlimb muscles that consequently display a similar pattern to diaphragm muscles [85]. In this model, punctures are performed randomly on the whole tibialis anterior muscle surface. In contrast to toxin injection, which triggers whole muscle damage, the micropins induced several local myofiber injuries that, when repeated daily, trigger chronic injury. A more physiological method has been to use exercise training, which is known to exacerbate the process of muscle degeneration/regeneration by increasing fiber necrosis and amplifying inflammatory reactions [86]. After one month of training, fibrogenesis is induced in hindlimb muscles and is further aggravated by a prolonged exercise regime. Alternative and faster ways to trigger fibrosis in the limb muscle of mdx mice are based on surgical and chemical damage, such as laceration or denervation. Laceration consists in a deep cut across the muscle [87] which causes a delay in the healing process, while the denervation model involves severing the sciatic nerve, thus causing atrophy of the denervated myofibers [88]. These are two powerful methods for inducing sustained fibrosis, the disadvantage being the limited area of the muscle affected and thus available for sampling, or the number of muscles affected, respectively.

7. Treatment of Fibrosis and Clinical Perspectives for DMD

Fibrosis development is a consequence of the chronic degeneration and impaired regeneration of dystrophic muscle, which is itself caused by loss of the dystrophin gene. While the core aim of gene or cell therapy remains to replace the missing gene and thereby cure the disease at the roots by targeting the cause of muscle degeneration, preventing fibrosis progression should be considered an adjunct therapy for several reasons. Firstly, fibrosis development may negatively interfere with cell and gene therapies by reducing the amount of target tissue available for repair. Secondly, preventing fibrosis can also potentially improve quality of life and lifespan of dystrophic patients on its own. Several pharmacological treatments targeting fibrotic cells or molecules are currently being tested and some are showing promising effects in human and animal models [89].

One of the signaling pathways involved in fibrosis development is the RAS system (as discussed above). Several components of this system have been used as targets to decrease dystrophic muscle fibrosis in animal models. Administration of the angiotensin 2 type 1 receptor antagonist losartan, which is commonly used to treat high blood pressure, has been shown to improve muscle strength and ameliorate fibrosis in dy(2J)/dy(2J) mice with laminin-α2-deficient congenital muscular dystrophy [90]. Other studies have also shown improved muscle function and diminished fibrosis in mice following losartan treatment associated with cardiotoxin injury and hindlimb immobilization-induced sarcopenia [91], decreased muscle fibrosis after laceration [92], and decreased cardiac fibrosis in mdx mice [93].

Other molecules target the RAS by inhibiting ACE. For example, lisinopril is an ACE-inhibitor that has been shown to preserve cardiac and skeletal muscle integrity in mdx mice [94]. Indeed, because of the positive preclinical effects shown for losartan and lisinopril, a recent double-blind randomized clinical trial was commenced to compare lisinopril versus losartan for the treatment of cardiomyopathy in human DMD patients [95]. Both drugs have already been shown to be effective for the treatment of dilated cardiomyopathy. Similarly to lisinopril, administration of the ACE inhibitor enalapril to mdx mice was shown to decrease skeletal muscle fibrosis [35]. Interestingly, treatment of mdx mice with the peptide angiotensin-1–7 (Ang 1–7) had the opposite effect to that of angiotensin 2, in that it improved muscle fibrosis by inhibiting TGFβ signaling and concomitantly decreasing the number of fibroblasts [40]. Taken together, these findings

suggest that targeting the RAS may be a promising way to delay fibrosis progression in DMD.

Targeting profibrotic growth factors or cytokines to slowdown fibrosis development has also showed promising results. Administration of the antibody FG-3019, which neutralizes CTGF, or the administration of an anti-TGFβ neutralizing antibody improves the phenotype of mdx mice by delaying fibrosis development [96]. However, as TGFβ and CTGF are pleiotropic, molecules, targeting them often induces undesired side effects such as increasing the amount of proinflammatory CD4+ T cells infiltrating the muscle [96].

Imatinib is an inhibitor of tyrosine kinase receptors, including PDGFRα, which was used originally for human cancer therapy. It has been shown that its administration is beneficial for muscular dystrophy in mdx mice [97]. Interestingly, imatinib appears to target specifically mesenchymal progenitors by inhibiting both their proliferation and expression of fibrosis markers *in vitro* [98]. Other molecules, known to have anti-inflammatory effects, have also shown beneficial effects by delaying fibrosis progression in mdx muscle, including halofuginone, a synthetic halogenated derivative of the naturally occurring molecule febrifugine [99]. Halofuginone was shown to greatly improve muscle histopathology and fibrosis in a model of dysferlin deficient mice [100]. Interestingly, halofuginone is known to inhibit specifically Th17 cell differentiation [101], suggesting that this molecule might act by modulating inflammation in the dystrophic muscle. Indeed, a recently concluded clinical trial (reference NCT01847573, http://clinicaltrials.gov/show/NCT01847573) has assessed the safety and tolerability of halofuginone in DMD patients, but no data on efficacy will be available for some time. However, despite several drugs showing promise in animal models, few have currently progressed to clinical trials in DMD patients.

8. Concluding Remarks and Perspectives

Fibrosis is an excessive deposition of ECM components that sometimes occurs as a result of dysregulated or chronic damage and repair processes. Human DMD patients have characteristic signs of muscle necrosis and repair with persistent fibrosis in muscles at young age, which play a significant role in the progressive nature of the disease and the reduced life expectancy. However, the mdx mouse model of DMD does not normally develop fibrosis extensively in the limbs, particularly at young age, and has several other differences from the human disease. Here we have reviewed some of the recent literature that has tried to bridge this species difference in order to develop better models to study the human condition, to improve existing treatments, and to open the door for new ones. Recent animal models have been shown to be better tools for unraveling the roles of new cell mediators in repair and fibrosis, including inflammatory and mesenchymal cell subpopulations, but the identity of a real ECM-producing cell in skeletal muscle remains elusive. Moreover, cell sorting techniques in mice are often complicated to reproduce in human patients due to differences in cell surface markers and the unavailability of sufficient sample material to extract significant numbers of cells. Advances have been made in the identification of new growth factors and cytokines and their downstream signaling components, which are also important targets for ameliorating fibrosis. As well as new advances in targeting TGFβ with antibodies, the more recent implication of the RAS system in fibrosis may also prove important mechanisms as there are already many approved drugs on the market that are able to modulate this system and potentially produce beneficial effects. However, despite all the promises shown in treating mice, there have been few clinical trials to date. While this is frustrating to patients and families, there is also a need for caution in advancing too quickly since there are still many unknowns and many clear differences in muscle pathology and fibrosis development between human DMD patients and mdx mice that may respond unexpectedly to treatment. Nonetheless, these differences are growing smaller as new animal models and new molecular tools are developed.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors thank C. Mann and the members of the Cell Biology Group for their helpful discussions and acknowledge funding from MINECO-Spain (SAF2012-38547, FIS-PS09/01267, FIS-PI13/025, and PLE2009-0124), AFM, E-Rare, Fundació MaratóTV3, Duchenne PP-NL, MDA, and EU-FP7 (Myoage, Optistem, and Endostem). Yacine Kharraz and Patrizia Pessina were supported by postdoctoral fellowships from AFM.

References

[1] A. E. H. Emery, "The muscular dystrophies," *The Lancet*, vol. 359, no. 9307, pp. 687–695, 2002.

[2] D. G. Leung and K. R. Wagner, "Therapeutic advances in muscular dystrophy," *Annals of Neurology*, vol. 74, pp. 404–411, 2013.

[3] U. T. Ruegg, "Pharmacological prospects in the treatment of Duchenne muscular dystrophy," *Current Opinion in Neurology*, vol. 26, pp. 577–584, 2013.

[4] M. Meregalli, A. Farini, M. Belicchi et al., "Perspectives of stem cell therapy in Duchenne muscular dystrophy," *FEBS Journal*, vol. 280, pp. 4251–4262, 2013.

[5] S. Benedetti, H. Hoshiya, and F. S. Tedesco, "Repair or replace? Exploiting novel gene and cell therapy strategies for muscular dystrophies," *FEBS Journal*, vol. 280, pp. 4263–4280, 2013.

[6] D. Briggs and J. E. Morgan, "Recent progress in satellite cell/myoblast engraftment—relevance for therapy," *FEBS Journal*, vol. 280, pp. 4281–4293, 2013.

[7] P. Konieczny, K. Swiderski, and J. S. Chamberlain, "Gene and cell-mediated therapies for muscular dystrophy," *Muscle & Nerve*, vol. 47, pp. 649–663, 2013.

[8] C. J. Mann, E. Perdiguero, Y. Kharraz et al., "Aberrant repair and fibrosis development in skeletal muscle," *Skeletal Muscle*, vol. 1, article 21, 2011.

[9] J. Schessl, Y. Zou, and C. G. Bönnemann, "Congenital muscular dystrophies and the extracellular matrix," *Seminars in Pediatric Neurology*, vol. 13, no. 2, pp. 80–89, 2006.

[10] E. M. MacDonald and R. D. Cohn, "TGFbeta signaling: its role in fibrosis formation and myopathies," *Current Opinion in Rheumatology*, vol. 24, pp. 628–634, 2012.

[11] R. L. Lieber and S. R. Ward, "Cellular mechanisms of tissue fibrosis—4. Structural and functional consequences of skeletal muscle fibrosis," *American Journal of Physiology. Cell Physiology*, vol. 305, pp. C241–C252, 2013.

[12] A. L. Serrano and P. Muñoz-Cánoves, "Regulation and dysregulation of fibrosis in skeletal muscle," *Experimental Cell Research*, vol. 316, no. 18, pp. 3050–3058, 2010.

[13] T. Bowen, R. H. Jenkins, and D. J. Fraser, "MicroRNAs, transforming growth factor beta-1, and tissue fibrosis," *Journal of Pathology*, vol. 229, pp. 274–285, 2013.

[14] L. Zhou, J. D. Porter, G. Cheng et al., "Temporal and spatial mRNA expression patterns of TGF-β1, 2, 3 and TβRI, II, III in skeletal muscles of mdx mice," *Neuromuscular Disorders*, vol. 16, no. 1, pp. 32–38, 2006.

[15] M. Horiguchi, M. Ota, and D. B. Rifkin, "Matrix control of transforming growth factor-beta function," *Journal of Biochemistry*, vol. 152, pp. 321–329, 2013.

[16] B. Vidal, A. L. Serrano, M. Tjwa et al., "Fibrinogen drives dystrophic muscle fibrosis via a TGFβ/alternative macrophage activation pathway," *Genes and Development*, vol. 22, no. 13, pp. 1747–1752, 2008.

[17] E. Ardite, E. Perdiguero, B. Vidal, S. Gutarra, A. L. Serrano, and P. Muñoz-Cánoves, "PAI-1-regulated miR-21 defines a novel age-associated fibrogenic pathway in muscular dystrophy," *Journal of Cell Biology*, vol. 196, no. 1, pp. 163–175, 2012.

[18] T. A. Wynn, "Cellular and molecular mechanisms of fibrosis," *Journal of Pathology*, vol. 214, no. 2, pp. 199–210, 2008.

[19] R. Samarakoon, A. D. Dobberfuhl, C. Cooley et al., "Induction of renal fibrotic genes by TGF-beta1 requires EGFR activation, p53 and reactive oxygen species," *Cellular Signalling*, vol. 25, pp. 2198–2209, 2013.

[20] T. A. Wynn, "Common and unique mechanisms regulate fibrosis in various fibroproliferative diseases," *Journal of Clinical Investigation*, vol. 117, no. 3, pp. 524–529, 2007.

[21] Y. Shi and J. Massagué, "Mechanisms of TGF-β signaling from cell membrane to the nucleus," *Cell*, vol. 113, no. 6, pp. 685–700, 2003.

[22] X. Shi-Wen, S. K. Parapuram, D. Pala et al., "Requirement of transforming growth factor β-activated kinase 1 for transforming growth factor β-induced α-smooth muscle actin expression and extracellular matrix contraction in fibroblasts," *Arthritis and Rheumatism*, vol. 60, no. 1, pp. 234–241, 2009.

[23] Y. Dong, R. Lakhia, S. S. Thomas, X. H. Wang, K. A. Silva, and L. Zhang, "Interactions between p-Akt and Smad3 in injured muscles initiate myogenesis or fibrogenesis," *American Journal of Physiology. Endocrinology and Metabolism*, vol. 305, pp. E367–E375, 2013.

[24] E. Brandan, C. Cabello-Verrugio, and C. Vial, "Novel regulatory mechanisms for the proteoglycans decorin and biglycan during muscle formation and muscular dystrophy," *Matrix Biology*, vol. 27, no. 8, pp. 700–708, 2008.

[25] Y. Li, W. Foster, B. M. Deasy et al., "Transforming growth factor-beta1 induces the differentiation of myogenic cells into fibrotic cells in injured skeletal muscle: a key event in muscle fibrogenesis," *American Journal of Pathology*, vol. 164, no. 3, pp. 1007–1019, 2004.

[26] Y. Li, J. Li, J. Zhu et al., "Decorin gene transfer promotes muscle cell differentiation and muscle regeneration," *Molecular Therapy*, vol. 15, no. 9, pp. 1616–1622, 2007.

[27] J. Narola, S. N. Pandey, A. Glick, and Y. W. Chen, "Conditional expression of TGF-beta1 in skeletal muscles causes endomysial fibrosis and myofibers atrophy," *PLoS ONE*, vol. 8, Article ID e79356, 2013.

[28] A. Heydemann, E. Ceco, J. E. Lim et al., "Latent TGF-β-binding protein 4 modifies muscular dystrophy in mice," *Journal of Clinical Investigation*, vol. 119, no. 12, pp. 3703–3712, 2009.

[29] G. Sun, K. Haginoya, Y. Wu et al., "Connective tissue growth factor is overexpressed in muscles of human muscular dystrophy," *Journal of the Neurological Sciences*, vol. 267, no. 1-2, pp. 48–56, 2008.

[30] M. G. Morales, J. Gutierrez, C. Cabello-Verrugio et al., "Reducing CTGF/CCN2 slows down mdx muscle dystrophy and improves cell therapy," *Human Molecular Genetics*, vol. 22, pp. 4938–4951, 2013.

[31] K. Frazier, S. Williams, D. Kothapalli, H. Klapper, and G. R. Grotendorst, "Stimulation of fibroblast cell growth, matrix production, and granulation tissue formation by connective tissue growth factor," *Journal of Investigative Dermatology*, vol. 107, no. 3, pp. 404–411, 1996.

[32] M. G. Morales, C. Cabello-Verrugio, C. Santander, D. Cabrera, R. Goldschmeding, and E. Brandan, "CTGF/CCN-2 overexpression can directly induce features of skeletal muscle dystrophy," *Journal of Pathology*, vol. 225, no. 4, pp. 490–501, 2011.

[33] C. Vial, J. Gutiérrez, C. Santander, D. Cabrera, and E. Brandan, "Decorin interacts with connective tissue growth factor (CTGF)/CCN2 by LRR12 inhibiting its biological activity," *Journal of Biological Chemistry*, vol. 286, no. 27, pp. 24242–24252, 2011.

[34] C. Vial, L. M. Zúñiga, C. Cabello-Verrugio, P. Cañón, R. Fadic, and E. Brandan, "Skeletal muscle cells express the profibrotic cytokine connective tissue growth factor (CTGF/CCN2), which induces their dedifferentiation," *Journal of Cellular Physiology*, vol. 215, no. 2, pp. 410–421, 2008.

[35] M. G. Morales, D. Cabrera, C. Cespedes et al., "Inhibition of the angiotensin-converting enzyme decreases skeletal muscle fibrosis in dystrophic mice by a diminution in the expression and activity of connective tissue growth factor (CTGF/CCN-2)," *Cell and Tissue Research*, vol. 353, pp. 173–187, 2013.

[36] C. P. Vío and V. A. Jeanneret, "Local induction of angiotensin-converting enzyme in the kidney as a mechanism of progressive renal diseases," *Kidney International*, vol. 64, no. 86, pp. S57–S63, 2003.

[37] G. Sun, K. Haginoya, H. Dai et al., "Intramuscular renin-angiotensin system is activated in human muscular dystrophy," *Journal of the Neurological Sciences*, vol. 280, no. 1-2, pp. 40–48, 2009.

[38] C. Cabello-Verrugio, M. J. Acuña, M. G. Morales, A. Becerra, F. Simon, and E. Brandan, "Fibrotic response induced by angiotensin-II requires NAD(P)H oxidase-induced reactive oxygen species (ROS) in skeletal muscle cells," *Biochemical and Biophysical Research Communications*, vol. 410, no. 3, pp. 665–670, 2011.

[39] P. Painemal, M. J. Acuna, C. Riquelme, E. Brandan, and C. Cabello-Verrugio, "Transforming growth factor type beta 1 increases the expression of angiotensin II receptor type 2 by a SMAD- and p38 MAPK-dependent mechanism in skeletal muscle," *Biofactors*, vol. 39, pp. 467–475, 2013.

[40] M. J. Acuna, P. Pessina, H. Olguin et al., "Restoration of muscle strength in dystrophic muscle by angiotensin-1-7 through inhibition of TGF-beta signalling," *Human Molecular Genetics*, vol. 23, pp. 1237–1249, 2014.

[41] X. Chen and Y. Li, "Role of matrix metalloproteinases in skeletal muscle: migration, differentiation, regeneration and fibrosis," *Cell Adhesion & Migration*, vol. 3, no. 4, pp. 337–341, 2009.

[42] Y. Nagamine, R. L. Medcalf, and P. Muñoz-Cánoves, "Transcriptional and posttranscriptional regulation of the plasminogen activator system," *Thrombosis and Haemostasis*, vol. 93, no. 4, pp. 661–675, 2005.

[43] M. Suelves, B. Vidal, V. Ruiz et al., "The plasminogen activation system in skeletal muscle regeneration: antagonistic roles of Urokinase-type Plasminogen Activator (UPA) and its inhibitor (PAI-1)," *Frontiers in Bioscience*, vol. 10, no. 3, pp. 2978–2985, 2005.

[44] Y. Ohtake, H. Tojo, and M. Seiki, "Multifunctional roles of MT1-MMP in myofiber formation and morphostatic maintenance of skeletal muscle," *Journal of Cell Science*, vol. 119, no. 18, pp. 3822–3832, 2006.

[45] R. Visse and H. Nagase, "Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry," *Circulation Research*, vol. 92, no. 8, pp. 827–839, 2003.

[46] M. Suelves, B. Vidal, A. L. Serrano et al., "uPA deficiency exacerbates muscular dystrophy in MDX mice," *Journal of Cell Biology*, vol. 178, no. 6, pp. 1039–1051, 2007.

[47] S. Zanotti, T. Negri, C. Cappelletti et al., "Decorin and biglycan expression is differentially altered in several muscular dystrophies," *Brain*, vol. 128, no. 11, pp. 2546–2555, 2005.

[48] L. Arnold, A. Henry, F. Poron et al., "Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis," *Journal of Experimental Medicine*, vol. 204, no. 5, pp. 1057–1069, 2007.

[49] Y. Kharraz, J. Guerra, C. J. Mann, A. L. Serrano, and P. Munoz-Canoves, "Macrophage plasticity and the role of inflammation in skeletal muscle repair," *Mediators of Inflammation*, vol. 2013, Article ID 491497, 9 pages, 2013.

[50] M. Wehling, M. J. Spencer, and J. G. Tidball, "A nitric oxide synthase transgene ameliorates muscular dystrophy in mdx mice," *Journal of Cell Biology*, vol. 155, no. 1, pp. 123–131, 2001.

[51] S. Gordon, "Alternative activation of macrophages," *Nature Reviews Immunology*, vol. 3, no. 1, pp. 23–35, 2003.

[52] T. A. Wynn, "Fibrotic disease and the TH1/TH2 paradigm," *Nature Reviews Immunology*, vol. 4, no. 8, pp. 583–594, 2004.

[53] I. Desguerre, C. Christov, M. Mayer et al., "Clinical heterogeneity of Duchenne muscular dystrophy (DMD): definition of sub-phenotypes and predictive criteria by long-term follow-up," *PLoS ONE*, vol. 4, no. 2, Article ID e4347, 2009.

[54] S. A. Villalta, H. X. Nguyen, B. Deng, T. Gotoh, and J. G. Tidbal, "Shifts in macrophage phenotypes and macrophage competition for arginine metabolism affect the severity of muscle pathology in muscular dystrophy," *Human Molecular Genetics*, vol. 18, no. 3, pp. 482–496, 2009.

[55] M. Wehling-Henricks, M. C. Jordan, T. Gotoh, W. W. Grody, K. P. Roos, and J. G. Tidball, "Arginine metabolism by macrophages promotes cardiac and muscle fibrosis in mdx muscular dystrophy," *PloS ONE*, vol. 5, no. 5, Article ID e10763, 2010.

[56] J. K. Ryu, D. Davalos, and K. Akassoglou, "Fibrinogen signal transduction in the nervous system," *Journal of Thrombosis and Haemostasis*, vol. 7, no. 1, pp. 151–154, 2009.

[57] B. Vidal, E. Ardite, M. Suelves et al., "Amelioration of Duchenne muscular dystrophy in mdx mice by elimination of matrix-associated fibrin-driven inflammation coupled to the $\alpha m\beta 2$ leukocyte integrin receptor," *Human Molecular Genetics*, vol. 21, no. 9, pp. 1989–2004, 2012.

[58] A. Farini, M. Meregalli, M. Belicchi et al., "T and B lymphocyte depletion has a marked effect on the fibrosis of dystrophic skeletal muscles in the scid/mdx mouse," *Journal of Pathology*, vol. 213, no. 2, pp. 229–238, 2007.

[59] J. Morrison, Q. L. Lu, C. Pastoret, T. Partridge, and G. Bou-Gharios, "T-cell-dependent fibrosis in the mdx dystrophic mouse," *Laboratory Investigation*, vol. 80, no. 6, pp. 881–891, 2000.

[60] M. J. Spencer, E. Montecino-Rodriguez, K. Dorshkind, and J. G. Tidball, "Helper (CD4+) and cytotoxic (CD8+) T cells promote the pathology of dystrophin-deficient muscle," *Clinical Immunology*, vol. 98, no. 2, pp. 235–243, 2001.

[61] S. A. Vetrone, E. Montecino-Rodriguez, E. Kudryashova et al., "Osteopontin promotes fibrosis in dystrophic mouse muscle by modulating immune cell subsets and intramuscular TGF-β," *Journal of Clinical Investigation*, vol. 119, no. 6, pp. 1583–1594, 2009.

[62] C. N. Pagel, D. K. Wasgewatte Wijesinghe, N. Taghavi Esfandouni, and E. J. Mackie, "Osteopontin, inflammation and myogenesis: influencing regeneration, fibrosis and size of skeletal muscle," *Journal of Cell Communication and Signaling*, 2013.

[63] L. Barron and T. A. Wynn, "Fibrosis is regulated by Th2 and Th17 responses and by dynamic interactions between fibroblasts and macrophages," *American Journal of Physiology. Gastrointestinal and Liver Physiology*, vol. 300, no. 5, pp. G723–G728, 2011.

[64] S. Lo Re, D. Lison, and F. Huaux, "CD4+ T lymphocytes in lung fibrosis: diverse subsets, diverse functions," *Journal of Leukocyte Biology*, vol. 93, pp. 499–510, 2013.

[65] A. W. B. Joe, L. Yi, A. Natarajan et al., "Muscle injury activates resident fibro/adipogenic progenitors that facilitate myogenesis," *Nature Cell Biology*, vol. 12, no. 2, pp. 153–163, 2010.

[66] A. Uezumi, S.-I. Fukada, N. Yamamoto, S. Takeda, and K. Tsuchida, "Mesenchymal progenitors distinct from satellite cells contribute to ectopic fat cell formation in skeletal muscle," *Nature Cell Biology*, vol. 12, no. 2, pp. 143–152, 2010.

[67] M. M. Murphy, J. A. Lawson, S. J. Mathew, D. A. Hutcheson, and G. Kardon, "Satellite cells, connective tissue fibroblasts and their interactions are crucial for muscle regeneration," *Development*, vol. 138, no. 17, pp. 3625–3637, 2011.

[68] S. J. Mathew, J. M. Hansen, A. J. Merrell et al., "Connective tissue fibroblasts and Tcf4 regulate myogenesis," *Development*, vol. 138, no. 2, pp. 371–384, 2011.

[69] R. N. Judson, R. H. Zhang, and F. M. Rossi, "Tissue-resident mesenchymal stem/progenitor cells in skeletal muscle: collaborators or saboteurs?" *FEBS Journal*, vol. 280, pp. 4100–4108, 2013.

[70] A. Uezumi, T. Ito, D. Morikawa et al., "Fibrosis and adipogenesis originate from a common mesenchymal progenitor in skeletal muscle," *Journal of Cell Science*, vol. 124, no. 21, pp. 3654–3664, 2011.

[71] S. Dulauroy, S. E. di Carlo, F. Langa, G. Eberl, and L. Peduto, "Lineage tracing and genetic ablation of ADAM12(+) perivascular cells identify a major source of profibrotic cells during acute tissue injury," *Nature Medicine*, vol. 18, pp. 1262–1270, 2012.

[72] L. H. Jørgensen, C. H. Jensen, U. M. Wewer, and H. D. Schrøder, "Transgenic overexpression of ADAM12 suppresses muscle regeneration and aggravates dystrophy in aged mdx mice," *American Journal of Pathology*, vol. 171, no. 5, pp. 1599–1607, 2007.

[73] V. S. LeBleu, G. Taduri, J. O'Connell et al., "Origin and function of myofibroblasts in kidney fibrosis," *Nature Medicine*, vol. 19, pp. 1047–1053, 2013.

[74] S. M. Wu, K. R. Chien, and C. Mummery, "Origins and fates of cardiovascular progenitor cells," *Cell*, vol. 132, no. 4, pp. 537–543, 2008.

[75] A. S. Brack, M. J. Conboy, S. Roy et al., "Increased Wnt signaling during aging alters muscle stem cell fate and increases fibrosis," *Science*, vol. 317, no. 5839, pp. 807–810, 2007.

[76] P. Zordan, E. Rigamonti, K. Freudenberg et al., "Macrophages commit postnatal endothelium-derived progenitors to angiogenesis and restrict endothelial to mesenchymal transition during muscle regeneration," *Cell Death & Disease*, vol. 5, Article ID e1031, 2014.

[77] J. W. Carnwath and D. M. Shotton, "Muscular dystrophy in the mdx mouse: histopathology of the soleus and extensor digitorum longus muscles," *Journal of the Neurological Sciences*, vol. 80, no. 1, pp. 39–54, 1987.

[78] G. B. Banks and J. S. Chamberlain, "The value of mammalian models for duchenne muscular dystrophy in developing therapeutic strategies," *Current Topics in Developmental Biology*, vol. 84, pp. 431–453, 2008.

[79] L. Zhou, J. A. Rafael-Fortney, P. Huang et al., "Haploinsufficiency of utrophin gene worsens skeletal muscle inflammation and fibrosis in mdx mice," *Journal of the Neurological Sciences*, vol. 264, no. 1-2, pp. 106–111, 2008.

[80] A. Sacco, F. Mourkioti, R. Tran et al., "Short telomeres and stem cell exhaustion model duchenne muscular dystrophy in mdx/mTR mice," *Cell*, vol. 143, no. 7, pp. 1059–1071, 2010.

[81] F. Mourkioti, J. Kustan, P. Kraft et al., "Role of telomere dysfunction in cardiac failure in Duchenne muscular dystrophy," *Nature Cell Biology*, vol. 15, pp. 895–904, 2013.

[82] K. Chandrasekharan, J. H. Yoon, Y. Xu et al., "A human-specific deletion in mouse Cmah increases disease severity in the mdx model of duchenne muscular dystrophy," *Science Translational Medicine*, vol. 2, no. 42, Article ID 42ra54, 2010.

[83] P. T. Martin, M. Camboni, R. Xu et al., "N-Glycolylneuraminic acid deficiency worsens cardiac and skeletal muscle pathophysiology in alpha-sarcoglycan-deficient mice," *Glycobiology*, vol. 23, pp. 833–843, 2013.

[84] S.-I. Fukada, D. Morikawa, Y. Yamamoto et al., "Genetic background affects properties of satellite cells and mdx phenotypes," *American Journal of Pathology*, vol. 176, no. 5, pp. 2414–2424, 2010.

[85] I. Desguerre, L. Arnold, A. Vignaud et al., "A new model of experimental fibrosis in hindlimb skeletal muscle of adult mdx mouse mimicking muscular dystrophy," *Muscle Nerve*, vol. 45, pp. 803–814, 2012.

[86] A. de Luca, S. Pierno, A. Liantonio et al., "Enhanced dystrophic progression in mdx mice by exercise and beneficial effects of taurine and insulin-like growth factor-1," *Journal of Pharmacology and Experimental Therapeutics*, vol. 304, no. 1, pp. 453–463, 2003.

[87] J. Menetrey, C. Kasemkijwattana, F. H. Fu, M. S. Moreland, and J. Huard, "Suturing versus immobilization of a muscle laceration. A morphological and functional study in a mouse model," *American Journal of Sports Medicine*, vol. 27, no. 2, pp. 222–229, 1999.

[88] B. M. Carlson, L. Billington, and J. Faulkner, "Studies on the regenerative recovery of long-term denervated muscle in rats," *Restorative Neurology and Neuroscience*, vol. 10, no. 2, pp. 77–84, 1996.

[89] H. Abdel-Hamid and P. R. Clemens, "Pharmacological therapies for muscular dystrophies," *Current Opinion in Neurology*, vol. 25, pp. 604–608, 2012.

[90] M. Elbaz, N. Yanay, S. Aga-Mizrachi et al., "Losartan, a therapeutic candidate in congenital muscular dystrophy: studies in the dy2J/dy2J mouse," *Annals of Neurology*, vol. 71, no. 5, pp. 699–708, 2012.

[91] T. N. Burks, E. Andres-Mateos, R. Marx et al., "Losartan restores skeletal muscle remodeling and protects against disuse atrophy in sarcopenia," *Science Translational Medicine*, vol. 3, no. 82, Article ID 82ra37, 2011.

[92] J. K. Park, M. R. Ki, E. M. Lee et al., "Losartan improves adipose tissue-derived stem cell niche by inhibiting transforming growth factor-beta and fibrosis in skeletal muscle injury," *Cell Transplantation*, vol. 21, pp. 2407–2424, 2012.

[93] L. T. Bish, M. Yarchoan, M. M. Sleeper et al., "Chronic losartan administration reduces mortality and preserves cardiac but not skeletal muscle function in dystrophic mice," *PLoS ONE*, vol. 6, no. 6, Article ID e20856, 2011.

[94] J. A. Rafael-Fortney, N. S. Chimanji, K. E. Schill et al., "Early treatment with lisinopril and spironolactone preserves cardiac and skeletal muscle in duchenne muscular dystrophy mice," *Circulation*, vol. 124, no. 5, pp. 582–588, 2011.

[95] H. D. Allen, K. M. Flanigan, and P. T. Thrush, "A randomized, double-blind trial of lisinopril and losartan for the treatment of cardiomyopathy in duchenne muscular dystrophy," *PLoS Currents*, 2013.

[96] F. Andreetta, P. Bernasconi, F. Baggi et al., "Immunomodulation of TGF-beta1 in mdx mouse inhibits connective tissue proliferation in diaphragm but increases inflammatory response: implications for antifibrotic therapy," *Journal of Neuroimmunology*, vol. 175, no. 1-2, pp. 77–86, 2006.

[97] J. C. D. S. Bizario, D. G. Cerri, L. C. Rodrigues et al., "Imatinib mesylate ameliorates the dystrophic phenotype in exercised mdx mice," *Journal of Neuroimmunology*, vol. 212, no. 1-2, pp. 93–101, 2009.

[98] T. Ito, R. Ogawa, A. Uezumi et al., "Imatinib attenuates severe mouse dystrophy and inhibits proliferation and fibrosis-marker expression in muscle mesenchymal progenitors," *Neuromuscular Disorders*, vol. 23, pp. 349–356, 2013.

[99] M. Pines and O. Halevy, "Halofuginone and muscular dystrophy," *Histology and Histopathology*, vol. 26, no. 1, pp. 135–146, 2011.

[100] K. D. Huebner, D. S. Jassal, O. Halevy, M. Pines, and J. E. Anderson, "Functional resolution of fibrosis in mdx mouse dystrophic heart and skeletal muscle by halofuginone," *American Journal of Physiology. Heart and Circulatory Physiology*, vol. 294, no. 4, pp. H1550–H1561, 2008.

[101] M. S. Sundrud, S. B. Koralov, M. Feuerer et al., "Halofuginone inhibits th17 cell differentiation by activating the amino acid starvation response," *Science*, vol. 324, no. 5932, pp. 1334–1338, 2009.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 168407, 7 pages
http://dx.doi.org/10.1155/2014/168407

Review Article

Inflammation Based Regulation of Cancer Cachexia

Jill K. Onesti[1,2] and Denis C. Guttridge[2,3]

[1] *Division of Surgical Oncology, The Ohio State University Wexner Medical Center, The Ohio State University College of Medicine, 460 W. 12th Avenue, Columbus, OH 43210, USA*
[2] *The Arthur G. James Comprehensive Cancer Center, Columbus, OH 43210, USA*
[3] *Human Cancer Genetics Program, Department of Molecular Virology, Immunology and Medical Genetics, The Ohio State University, Columbus, OH 43210, USA*

Correspondence should be addressed to Denis C. Guttridge; denis.guttridge@osumc.edu

Received 13 February 2014; Accepted 10 April 2014; Published 4 May 2014

Academic Editor: Dario Coletti

Cancer cachexia, consisting of significant skeletal muscle wasting independent of nutritional intake, is a major concern for patients with solid tumors that affects surgical, therapeutic, and quality of life outcomes. This review summarizes the clinical implications, background of inflammatory cytokines, and the origin and sources of procachectic factors including TNF-α, IL-6, IL-1, INF-γ, and PIF. Molecular mechanisms and pathways are described to elucidate the link between the immune response caused by the presence of the tumor and the final result of skeletal muscle wasting.

1. Clinical Significance of Cancer Cachexia

Cachexia associated with cancer leading to skeletal muscle wasting is a major cause of morbidity associated with numerous types of cancer. Varying definitions have been proposed to classify cachexia, but the central components include ongoing loss of muscle mass due to a negative protein balance [1–3]. Greater than 50% of patients with cancer have cachexia at the time of death, and more than 30% of patients die due to cachexia [4]. This has been shown to become increasingly worse as the cancer progresses, eventually reaching a limit with low likelihood of reversal [5]. Emerging evidence shows that skeletal muscle depletion in cancer patients is a powerful predictor of a worse overall prognosis across varying cancer etiologies [6–9].

Muscle atrophy/wasting, often used as a clinical marker of cachexia, has been shown to affect outcomes in patients undergoing surgery. The University of Michigan Analytical Morphomics Group has published their findings on the relationship between lean muscle mass and postoperative mortality in patients undergoing any major elective surgery (an increase in mortality by 45% for each 1000 mm^2 decrease in lean core muscle area) [9] which they found to be more predictive than chronological age [10]. This same pattern held true for patients with adrenocortical carcinoma [11] and melanoma [12]. The measurements for lean muscle mass were determined by measuring the cross-sectional area and Hounsfield units of the psoas muscle at the level of the fourth lumbar vertebra and excluding fatty infiltration.

Patients with operable cancer are greatly impacted by the presence of cachexia. This may be due to the fact that cachexia indicates a more advanced stage of tumor [13] or simply that the patient is overall frailer. In a study examining 557 patients undergoing pancreas resection for adenocarcinoma, Peng et al. found that muscle wasting was an independent factor associated with an increased risk of death at three years (HR = 1.63; $P < 0.001$) [14]. A similar finding was noted for patients undergoing hepatectomy for hepatocellular carcinoma (HR = 0.92; $P = 0.004$) [15]. Decreased muscle density was associated with an increased rate of complications but not overall outcomes for colon cancer in another study [16].

Not only are overall survival and surgical outcomes affected by cachexia but also quality of life. Several studies have shown that cachexia itself contributes to lower scores more so than tumor location, duration, or stage [17, 18].

Outward effects of cachexia include a decrease in physical activity and the ability to perform activities of daily living, which may play a role in a person's psychological well-being. These factors in turn lead to a lower performance status, negatively impacting the ability and availability of chemotherapeutic agents [18–22].

The theory of the origin of cancer cachexia is rooted in systemic inflammation and not solely reduction of nutritional intake [23], a reason why cachexia is now distinguished from anorexia (see below). Several easily identifiable factors have been studied in an attempt to quantify the degree of inflammation and use that data to predict outcomes or guide treatment. Elevated neutrophil : lymphocyte ratio (NLR) and C-reactive protein have been associated with low skeletal muscle mass [24] and early detection of cancer cachexia [25, 26]. NLR has been shown to predict outcomes in numerous types of solid [27–32]. Another scoring system, the modified Glasgow Prognostic Score, is based on C-reactive protein and albumin levels [33]. This has been shown to predict outcomes in patients with biliary [34], colorectal [35], prostate [36], and other tumors [37].

The effect of trying to reduce cachexia by avoiding bed rest and stimulating muscle exercise has met with limited results. A review by Stene and colleagues [38] summarized results from several randomized controlled trials examining cancer cachexia. They found that physical exercise may lead to reduced fatigue, improved quality of life, and decreased adverse effects, but further studies are needed to identify if there is any survival advantage, particularly in more advanced cancer stages.

2. Background of Inflammatory Cytokines

The clinical significance of cancer cachexia has been realized for some time. The imbalance between adequate caloric intake and total body energy expenditure has been the subject of research for several decades. Previous work has focused on the role of cytokines such as tumor necrosis factor-α (TNF-α), interleukins 1 and 6 (IL-1, IL-6), and interferon gamma (INF-γ).

A review article by Tisdale published in 1997 summarized the current literature at that time [39]. Cancer cachexia was noted to be different from simple starvation which strives to conserve muscle mass. In cancer cachexia, however, this conservation mechanism is missing, such that there is equal loss of adipose and muscular tissue. This finding highlights the fact that anorexia alone is not sufficient cause for cachexia, and, in fact, does not always precede it [40], nor is cachexia alleviated by the supplementation of intravenous hyperalimentation [41].

Probably more influential in the development of cachexia is the increase in energy expenditure due to an elevated basal metabolic rate [39]. This is associated with an elevated adrenergic state [42] and appears to be similar across tumor types. Many solid tumors have also been shown to have significantly elevated rates of carbohydrate metabolism [43, 44]. This increase in glucose utilization by the tumor translates into a lower supply for the host tissue. The primary site of lean body mass depletion is the skeletal muscle and this is due to an increased rate of protein turnover without an appropriately significant increase in protein synthesis [45]. The pathway regulating protein breakdown is the adenosine triphosphate- (ATP-) ubiquitin-dependent pathway. This has been shown to be upregulated in cancer cachexia [46] and the ATP-ubiquitin-dependent pathway appears to play a major role in cancer cachexia in weight loss up to 20% [47].

These responses are controlled, at least in part, by a variety of cytokines. TNF-α was initially thought to play a direct role in cachexia by inhibiting lipoprotein lipase and enhancing the protein degradation. The direct correlation between TNF-α levels and the degree of cachexia has been more difficult to prove, however [39]. Similarly, IL-1 has demonstrated some role in the cachexia pathway, but a direct mechanism for controlling tissue wasting has not been proven [39]. Increasing the levels of IL-6 has been shown to correlate with development of cachexia in certain mouse models [48, 49]. Treatments designed to bind to IL-6 and inhibit its effect have demonstrated improvement in cachexia [50, 51]. These results have also been demonstrated in human patients [52]. Studies have shown INF-γ to have similar properties to TNF-α in reducing body fat, but without an effect on total body protein [39]. Again, no association with the human clinical syndrome of cancer cachexia has been clearly elucidated.

The ubiquitin pathway is also regulated by a high affinity activin type 2 receptor (ActRIIB) [53]. Zhou et al. found that blockade of this pathway could reverse muscle loss and also led to prolonged survival in mice models of cancer cachexia. Interestingly, this reversal was not accompanied by a reduction in circulating levels of proinflammatory cytokines [53].

In a review by Argiles and Lopez-Soriano, cytokines are separated according to their function as either procachectic factors or anticachectic factors in order to further define their roles [54]. The procachectic factors include those mentioned above, which act by promoting tissue wasting. The anticachectic factors act in opposition by attempting to stabilize this breakdown. These factors include IL-4, IL-10, IL-12, IL-15, INF-α, and insulin-like growth factor I (IGF-I). These cytokines have been shown to ameliorate the effects of the procachectic factors to varying degrees, mostly in mouse models [54]. Clearly a balance must exist, and both procachectic and anticachectic factors are targets for clinical therapies.

3. Origins of Cachexia Mediators

Once the presence and function of cytokines in the pathogenesis of cachexia has been established, the origin and sources must be identified. Previous theories of the origin of cytokines have included the tumor itself versus the native host tissue [55].

Evidence for the release of cytokines from native host tissue is found in the presence of a persistent inflammatory response, mediated by T helper 1 (Th1) cells [55]. The presence of the tumor itself causes the body to produce an acute phase

response [56]. A review by de Visser and Coussens described how the body's innate immune system involves an increase in the local concentration of mast cells and macrophages leading to angiogenesis and tumor growth [57]. Mouse models of epithelial carcinogenesis have demonstrated that the absence of mast cells or the inability to recruit additional immune cells prohibits malignant transformation [58]. Macrophages appear to be the source of some of the principal mediators of cachexia, such as TNF-α or IL-1 [59]. Intriguingly, chronic inflammation may be associated with compromised immune function, such as an impaired T-cell response, via various inflammatory proteins, including sIL-2R, VEGF, and IL-17 [60], thus creating an environment even more permissive to tumor survival.

Certain myeloid immune suppressor cells have been found to promote tumor angiogenesis by the production of matrix metalloproteinase 9 (MMP-9) [61]. These factors even suggest that the presence of host immune cells is required for promoting neoplastic events [57]. Tumor infiltrating inflammatory cells also regulate angiogenesis as well as producing extracellular proteases that serve to remodel the extracellular environment allowing tumor potentiation and possibly even metastases [57, 62]. The authors make note that expression of MMP-9 primarily derives from host immune cells such as neutrophils, macrophages, and mast cells, as opposed to tumor cells [62].

One study found that a population of myeloid-derived suppressor cells grows dramatically within tumors, producing inappropriate quantities of inflammatory cytokines [63]. This increase was noted to be associated with cachexia. These cells and others of the innate immune system respond to tumors by producing TNF-α, IL-1β, IL-6, and INF-γ in an effort to stimulate the host's immune response and overcome any offending pathogens. As the cancer persists, however, the ongoing high inflammatory state begins to have ill effects towards the host, as well.

The specific role of IL-6 in cancer associated cachexia and skeletal muscle wasting has been identified [64]. In a study by White and colleagues, $Apc^{\mathrm{Min}/+}$ and wild type mice on a C57Bl/6 background were used to examine the effect of treatment with an IL-6 receptor antibody after the onset of cachexia as well as the effects of exercise [65]. They found that mitochondrial biogenesis was disrupted early in the development of cachexia, which could be rescued by administration of an IL-6 receptor antibody as well as exercise. Which factors downstream of IL-6 mediate effects on cachexia are still being elucidated but likely involve the transcription factor STAT3, which we describe in more detail below.

Tumor specific factors include proteolysis inducing factor (PIF) and lipid mobilizing factor (LMF), which serve to direct breakdown proteins and fat [55]. Increased concentrations of PIF have been identified in murine models consistent, and almost exclusively, with cancer cachexia [66] likely through the ATP-ubiquitin-dependent pathway [67]. In a study examining a human homologue of PIF, however, although elevated levels were noted in the presence of tumor, this alone was not enough to induce cachexia [68]. Another study found that PIF was expressed in patients with gastrointestinal tumors and that this expression correlated with weight loss [69].

The specific role of the tumor versus the host response is not always clearly delineated. Procachexia cytokines might be produced by the tumor as well as the host, whereas PIF appears to be produced exclusively by tumors [70]. In addition, PIF and TNF-α appear to induce muscle cachexia through a similar pathway, by activating the nuclear factor kappa B (NF-κB) transcription factor [71, 72]. Activation of this factor causes translocation to the nucleus where it binds to specific promoter regions, regulating the expression of proinflammatory cytokines [55] as well as the ubiquitin-proteasome pathway. Another pathway responsive to inflammation that was recently implemented in regulation of the ubiquitin-proteasome system is the CCAAT/enhancer binding protein beta (C/EBPβ) transcription factor whose activation depends on p38 MAP kinase.

Although PIF appears to clearly contribute to skeletal muscle loss in cancer cachexia, no other purely tumoral factor appears to have the same potential [70]. Therefore, the majority of mediators are due to the host's systemic response.

Another pathway that may contribute to cancer cachexia is autophagic degradation. The host's natural autophagic-lysosomal proteolysis may be altered in various pathologic states. In a study by Mizushima et al. autophagy was enhanced in skeletal muscle during the first 24 hours of starvation and sustained [73]. A direct link has also recently been described in cancer cachexia models, which showed that increased autophagic-lysosomal degradation is induced in cancer associated muscle atrophy and likely involves separate pathways from those involved in noncancer muscle wasting [74]. The FoxO transcription factors have been shown to function as strong transcriptional drivers of autophagic genes in response to cachectic factors [75].

4. Genetic Response to Cytokine Stimulation: STAT3 and Pax7

As described above, cytokines are important not only to establish tumor-host interaction and deregulate inflammatory response to tumor burden but also as mediators of muscle wasting by directly targeting muscle tissue. To this regard, cachexia appears to be a genetically regulated response, dependent on a specific subset of genes, which control a highly regulated process of muscle protein degradation [76]. Bonetto et al. described the process by which STAT3 is activated leading to an upregulation of the acute phase response [77]. IL-6 binds to the IL-6 reception α-chain, which causes dimerization and activation of associated Janus kinases. Two pathways are then activated, the STAT3 and the mitogen-activated protein kinase (MAPK/ERK) cascade. STAT3 then causes further dimerization and nuclear translocation and ultimately modulation of gene expression of the acute phase response. In their study, Bonetto et al. implanted colon-26 adenocarcinoma cells into Balb/c or CD2F1 mice. Mice were sacrificed after 19 and 24 days (10 and 15% weight loss, resp.) reflecting moderate and severe cachexia. Significant STAT3

activity was noted in gastrocnemius and quadriceps muscles. Mice were then injected with a recombinant adenovirus that constitutively expressed STAT3 and found significant elevation of fibrinogen levels, indicating that IL-6 activation of STAT3 is a potent stimulator of the acute phase response that leads to significant cachexia. It is worth noting that the authors found a low level of suppressor of cytokine signaling-3 (SOCS3) in this tumor model, which normally serves to inhibit STAT3 and self-regulate the duration of activation. This could explain how cachexia continues to persist despite clearly deleterious effects on the host.

STAT3 activation is not isolated to the IL-6 pathway, however. PIF has also been shown to activate STAT3 in hepatic cells, which also increases the production of proinflammatory cytokines leading to cachexia [78]. PIF has no other known function other than muscle degradation, but the authors theorize that its function could be critical during embryogenesis. Expression peaks during skeletal muscle and liver development in the developing fetus.

We and others have reported the observation of a massive upregulation of the muscle stem cell specification gene Pax7 in experimental models of cancer cachexia [79, 80]. Penna et al. inoculated Balb-c mice with colon-26 undifferentiated carcinoma. One group of mice was then injected with the MEK inhibitor PD98059. The mice were allowed free access to food and were sacrificed after 13 days. Significant muscle and body weight loss were observed, as was marked the phosphorylation of ERK, a mitogen activated protein kinase. Evidence for impaired myogenesis was noted in the tumor-bearing mice as evidenced by increased levels of Pax7. The degree of muscle wasting and Pax7 concentration were ameliorated by the injection of the MEK inhibitor PD98059, via inhibition of ERK. These findings supported the idea that satellite cells accumulate in muscle due to overproduction or impaired differentiation, leading to cachexia [79]. Similarly, elevated levels of Pax7 were found in skeletal muscle samples from patients with pancreatic cancer demonstrating cachexia [80]. This overexpression was shown to cause significant muscle atrophy due a block in the differentiation of muscle progenitor cells responding to injury signals emanating from the tumor. We found that the decreased levels of Pax7 could reverse the effects and allowed progenitor cells to differentiate and myofibers to be repaired [80]. Yet to be identified factors present in the serum of tumor-bearing mice are responsible for Pax7 upregulation and block of myogenic potential in muscle stem cells, a capacity not fully recapitulated by administration of specific, albeit important, recombinant cytokines, such as TNF-alpha [80]. This study not only pointed out for the first time the involvement of muscle stem cells in muscle wasting that does not merely consist of muscle fiber atrophy but also demonstrated that circulating factors have multiple targets in muscle and further extend their role in muscle homeostasis. Intriguingly, NF-κB was known for its role in response to inflammatory cytokines in many cell types including muscle [81, 82] and was previously demonstrated to be sufficient to trigger muscle atrophy [83, 84].

5. Clinical Trials

Several trials have been performed to identify the physiologic and clinical results of anticachexia treatment modalities in patients with advanced cancer. MacCiò et al. treated patients who had gynecological cancers with megestrol acetate plus l-carnitine, a COX-2 inhibitor (celecoxib), and antioxidants versus just megestrol acetate alone [85]. The combination treatment resulted in improvements in lean body mass, resting energy expenditure, fatigue, and quality of life. Proinflammatory cytokines and oxidative stress markers including IL-6, TNF-α, CRP, and reactive oxygen species (ROS) were decreased in the combination arm but were unchanged in the megestrol acetate alone arm.

A phase I/II study compared etanercept (an TNF-α blocker) with gemcitabine versus gemcitabine alone for treatment of patients with advanced pancreatic cancer [86]. Some clinical benefit was identified and was associated with IL-10 levels but did not show significant improvement in 6-month progression free survival compared to gemcitabine alone.

Similarly, a phase II trial compared the efficacy and safety of celecoxib on cancer cachexia [87]. All patients had advanced cancer of varying tumor sites. TNF-α levels were shown to decrease in the majority, and patients had a corresponding increase in lean body mass. However, changes in IL-6 levels were not significantly different after treatment.

6. Conclusions

Cancer cachexia is a very prevalent and debilitating aspect of solid tumors. In addition to predicting an overall worse prognosis, cachexia significantly decreases a patient's quality of life. Surgical outcomes are worsened, chemotherapeutics agents are limited, and daily activities are hindered.

The pathogenesis of cancer cachexia is highly dependent on the patient's immune response. Inflammatory cytokines, procachectic factors, induce muscle degradation even in the face of adequate nutrition. These cytokines are produced by the host in response to the tumor, as well as from tumor factors themselves. IL-6, TNF-α, and PIF are major contributors to the syndrome of muscle wasting.

The common pathway for muscle degradation involves the ubiquitin-proteasome pathway. Upstream activation is performed primarily through the NF-κB and STAT3 pathways, making them targets for potential interventions.

More research is essential to further elucidate and halt the dangerous progression of skeletal muscle breakdown in the face of solid tumors.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] O. Wallengren, K. Lundholm, and I. Bosaeus, "Diagnostic criteria of cancer cachexia: relation to quality of life, exercise

capacity, and survival in unselected palliative care patients," *Support Care Cancer*, vol. 21, no. 6, pp. 1569–1577, 2013.

[2] K. Fearon, F. Strasser, S. D. Anker et al., "Definition and classification of cancer cachexia: an international consensus," *The Lancet Oncology*, vol. 12, no. 5, pp. 489–495, 2011.

[3] L. Thoresen, G. Frykholm, S. Lydersen et al., "Nutritional status, cachexia and survival in patients with advanced colorectal carcinoma. Different assessment criteria for nutritional status provide unequal results," *Clinical Nutrition*, vol. 32, no. 1, pp. 65–72, 2013.

[4] S. von Haehling and S. D. Anker, "Cachexia as a major underestimated and unmet medical need: facts and numbers," *Journal of Cachexia, Sarcopenia and Muscle*, vol. 1, no. 1, pp. 1–5, 2010.

[5] C. M. Prado, M. B. Sawyer, S. Ghosh et al., "Central tenet of cancer cachexia therapy: do patients with advanced cancer have exploitable anabolic potential?" *The American Journal of Clinical Nutrition*, vol. 98, no. 4, pp. 1012–1019, 2013.

[6] L. Martin, L. Birdsell, N. Macdonald et al., "Cancer cachexia in the age of obesity: skeletal muscle depletion is a powerful prognostic factor, independent of body mass index," *Journal of Clinical Oncology*, vol. 31, no. 12, pp. 1539–1547, 2013.

[7] M. Trajkovic-Vidakovic, A. de Graeff, E. E. Voest, and S. C. C. M. Teunissen, "Symptoms tell it all: a systematic review of the value of symptom assessment to predict survival in advanced cancer patients," *Critical Reviews in Oncology/Hematology*, vol. 84, no. 1, pp. 130–148, 2012.

[8] B. H. L. Tan, L. A. Birdsell, L. Martin, V. E. Baracos, and K. C. H. Fearon, "Sarcopenia in an overweight or obese patient is an adverse prognostic factor in pancreatic cancer," *Clinical Cancer Research*, vol. 15, no. 22, pp. 6973–6979, 2009.

[9] M. J. Englesbe, J. S. Lee, K. He et al., "Analytic morphomics, core muscle size, and surgical outcomes," *Annals of Surgery*, vol. 256, no. 2, pp. 255–261, 2012.

[10] M. F. Englesbe, M. N. Terjimanian, J. S. Lee et al., "Morphometric age and surgical risk," *Journal of the American College of Surgeons*, vol. 216, no. 5, pp. 976–985, 2013.

[11] B. S. Miller, K. M. Ignatoski, S. Daignault et al., "Worsening central sarcopenia and increasing intra-abdominal fat correlate with decreased survival in patients with adrenocortical carcinoma," *World Journal of Surgery*, vol. 36, no. 7, pp. 1509–1516, 2012.

[12] M. S. Sabel, J. Lee, S. Cai, M. J. Englesbe, S. Holcombe, and S. Wang, "Sarcopenia as a prognostic factor among patients with stage III melanoma," *Annals of Surgical Oncology*, vol. 18, no. 13, pp. 3579–3585, 2011.

[13] J. Bachmann, K. Ketterer, C. Marsch et al., "Pancreatic cancerrelated cachexia: influence on metabolism and correlation to weight loss and pulmonary function," *BMC Cancer*, vol. 9, article 255, 2009.

[14] P. Peng, O. Hyder, A. Firoozmand et al., "Impact of sarcopenia on outcomes following resection of pancreatic adenocarcinoma," *Journal of Gastrointestinal Surgery*, vol. 16, no. 8, pp. 1478–1486, 2012.

[15] N. Harimoto, K. Shirabe, Y. I. Yamashita et al., "Sarcopenia as a predictor of prognosis in patients following hepatectomy for hepatocellular carcinoma," *British Journal of Surgery*, vol. 100, no. 11, pp. 1523–1530, 2013.

[16] M. S. Sabel, M. Terjimanian, A. S. C. Conlon et al., "Analytic morphometric assessment of patients undergoing colectomy for colon cancer," *Journal of Surgical Oncology*, vol. 108, no. 3, pp. 169–175, 2013.

[17] P. Ravasco, I. Monteiro-Grillo, P. Marques Vidal, and M. E. Camilo, "Cancer: disease and nutrition are key determinants of patients' quality of life," *Supportive Care in Cancer*, vol. 12, no. 4, pp. 246–252, 2004.

[18] V. C. Vaughan, P. Martin, and P. A. Lewandowski, "Cancer cacheia: impact, mechanisms and emerging treatments," *Journal of Cachexia, Sarcopenia and Muscle*, vol. 4, no. 2, pp. 95–109, 2013.

[19] C. M. M. Prado, V. E. Baracos, L. J. McCargar et al., "Body composition as an independent determinant of 5-fluorouracil-based chemotherapy toxicity," *Clinical Cancer Research*, vol. 13, no. 11, pp. 3264–3268, 2007.

[20] C. M. M. Prado, V. E. Baracos, L. J. McCargar et al., "Sarcopenia as a determinant of chemotherapy toxicity and time to tumor progression in metastatic breast cancer patients receiving capecitabine treatment," *Clinical Cancer Research*, vol. 15, no. 8, pp. 2920–2926, 2009.

[21] H. J. N. Andreyev, A. R. Norman, J. Oates, and D. Cunningham, "Why do patients with weight loss have a worse outcome when undergoing chemotherapy for gastrointestinal malignancies?" *European Journal of Cancer*, vol. 34, no. 4, pp. 503–509, 1998.

[22] S. Dalal, D. Hui, L. Bidaut et al., "Relationships among body mass index, longitudinal body composition alterations, and survival in patients with locally advanced pancreatic cancer receiving chemoradiation: a pilot study," *Journal of Pain and Symptom Management*, vol. 44, no. 2, pp. 181–191, 2012.

[23] T. S. Solheim, D. Blum, P. M. Fayers et al., "Weight loss, appetite loss and food intake in cancer patients with cancer cachexia: three peas in a pod?—analysis from a multicenter cross sectional study," *Acta Oncologica*, vol. 53, no. 4, pp. 539–546, 2014.

[24] C. H. Richards, C. S. D. Roxburgh, M. R. MacMilla et al., "The relationships between body composition and the systemic inflammatory response in patients with primary operable colorectal cancer," *PLoS ONE*, vol. 7, no. 8, Article ID e41883, 2012.

[25] B. Trutschnigg, R. D. Kilgour, J. A. Morais et al., "Metabolic, nutritional and inflammatory characteristics in elderly women with advanced cancer," *Journal of Geriatric Oncology*, vol. 4, no. 2, pp. 183–189, 2013.

[26] J. Wu, C. Huang, H. Xiao, Q. Tang, and W. Cai, "Weight loss and resting energy expenditure in male patients with newly diagnosed esophageal cancer," *Nutrition*, vol. 29, no. 11, pp. 1310–1314, 2013.

[27] G. Q. Xiao, C. Liu, D. L. Liu, J. Y. Yang, and L. N. Yan, "Neutrophil-lymphocyte ratio predicts the prognosis of patients with hepatocellular carcinoma after liver transplantation," *World Journal of Gastroenterology*, vol. 19, no. 45, pp. 8398–8407, 2013.

[28] Y. Jin, X. Ye, C. He, B. Zhang, and Y. Zhang, "Pretreatment neutrophil-to-lymphocyte ratio as predictor survival for patients with metastatic nasopharyngeal carcinoma," *Head & Neck*, 2014.

[29] L. Jankova, O. F. Dent, C. Chan, P. Chapuis, and S. J. Clarke, "Preoperative neutrophil/lymphocyte ratio predicts overall survival but does not predict recurrence or cancer-specific survival after curative resection of noe-positive colorectal cancer," *BMC Cancer*, vol. 13, article 442, 2013.

[30] T. Nakamura, A. Matsumine, T. MAtsubara, K. Asanuma, A. Uchida, and A. Sudo, "The combined use of the neutrophil-lymphocyte ratio and C-reactive protein level as prognostic predictors in adult patients with soft tissue sarcoma," *Journal of Surgical Oncology*, vol. 108, no. 7, pp. 481–485, 2013.

[31] H. Jin, G. Zhang, X. Liu et al., "Blood neutrophil-lymphocyte ratio predicts survival for stages III-IV gastric cancer treated with neoadjuvant chemotherapy," *World Journal of Surgical Oncology*, vol. 11, article 112, 2013.

[32] S. Dutta, A. B. C. Crumley, G. M. Fullarton, P. G. Horgan, and D. C. McMillan, "Comparison of the prognostic value of tumour- and patient-related factors in patients undergoing potentially curative resection of oesophageal cancer," *World Journal of Surgery*, vol. 35, no. 8, pp. 1861–1866, 2011.

[33] D. C. McMillan, "An inflammation-based prognostic score and its role in the nutrition-based management of patients with cancer," *Proceedings of the Nutrition Society*, vol. 67, no. 3, pp. 257–262, 2008.

[34] A. Iwaku, A. Kinoshita, H. Onoda, N. Fushiya, M. Matsushima, and H. Tajiri, "The Glasgow prognostic score accurately predicts survivial in patients with biliary tract cancer not indicated for surgical resection," *Medical Oncology*, vol. 31, no. 1, article 787, 2014.

[35] T. Kishiki, T. Masaki, H. Matsuoka et al., "Modified Glasgow prognositc score in patients with incurable stage IV colorectal cancer," *The American Journal of Surgery*, vol. 206, no. 2, pp. 234–240, 2013.

[36] K. Shafique, M. J. Proctor, D. C. McMillan et al., "The modified Glasgow prognostic score in prostate cancer: results from a retrospective clinical series of 744 patients," *BMC Cancer*, vol. 13, no. 1, article 292, 2013.

[37] D. C. McMillan, "The systemic inflammation-based Glasgow prognostic score: a decade of experience in patients with cancer," *Cancer Treatment Reviews*, vol. 39, no. 5, pp. 534–540, 2013.

[38] G. B. Stene, J. L. Helbostad, T. R. Blastad, I. I. Riphagen, S. Kaasa, and L. M. Oldrevoll, "Effect of physical exercise on muscle mass and strength in cancer patients during treatment—a systematic review," *Critical Reviews in Oncology/Hematology*, vol. 88, no. 3, pp. 573–593, 2013.

[39] M. J. Tisdale, "Biology of cachexia," *Journal of the National Cancer Institute*, vol. 89, no. 23, pp. 1763–1773, 1997.

[40] G. Costa, "Cachexia, the metabolic component of neoplastic diseases," *Cancer Research*, vol. 37, no. 7, pp. 2327–2335, 1977.

[41] W. K. Evans, R. Makuch, G. H. Clamon et al., "Limited impact of total parenteral nutrition on nutritional status during treatment for small cell lung cancer," *Cancer Research*, vol. 45, no. 7, pp. 3347–3353, 1985.

[42] C. Drott, G. Svaninger, and K. Lundholm, "Increased urinary excretion of cortisol and catecholami-NES in malnourished cancer patients," *Annals of Surgery*, vol. 208, no. 5, pp. 645–650, 1988.

[43] E. Holm, E. Hagmuller, U. Staedt et al., "Substrate balances across colonic carcinomas in humans," *Cancer Research*, vol. 55, no. 6, pp. 1373–1378, 1995.

[44] H. D. Mulligan and M. J. Tisdale, "Metabolic substrate utilization by tumour and host tissues in cancer cachexia," *Biochemical Journal*, vol. 277, no. 2, pp. 321–326, 1991.

[45] P. W. Emery, R. H. T. Edwards, and M. J. Rennie, "Protein synthesis in muscle measured in vivo in cachectic patients with cancer," *British Medical Journal*, vol. 289, no. 6445, pp. 584–586, 1984.

[46] M. Llovera, C. Garcia-Martinez, N. Agell, M. Marzabal, F. J. Lopez-Soriano, and J. M. Argiles, "Ubiquitin gen expression is increased in skeletal muscle of tumour-bearing rats," *FEBS Letters*, vol. 338, no. 3, pp. 311–318, 1994.

[47] J. Khal, S. M. Wyke, S. T. Russell, A. V. Hine, and M. J. Tisdale, "Expression of the ubiquitin-proteasome pathway and muscle loss in experimental cancer cachexia," *British Journal of Cancer*, vol. 93, no. 7, pp. 774–780, 2005.

[48] G. Strassmann, M. Fong, J. S. Kenney, and C. O. Jacob, "Evidence for the involvement of interleukin 6 in experimental cancer cachexia," *The Journal of Clinical Investigation*, vol. 89, no. 5, pp. 1681–1684, 1992.

[49] T. Tsujinaka, J. Fujita, C. Ebisui et al., "Interleukin 6 receptor antibody inhibits muscle atrophy and modulates proteolytic systems in interleukin 6 transgenic mice," *The Journal of Clinical Investigation*, vol. 97, no. 1, pp. 244–249, 1996.

[50] G. Strassmann, M. Fong, C. E. Freter, S. Windsor, F. D'Alessandro, and R. P. Nordan, "Suramin interferes with interleukin-6 receptor binding in vitro and inhibits colon-26-mediated experimental cancer cachexia in vivo," *The Journal of Clinical Investigation*, vol. 92, no. 5, pp. 2152–2159, 1993.

[51] S. Tamura, K. F. Ouchi, K. Mori et al., "Involvement of human interleukin 6 in experimental cachexia induced by a human uterine cervical carcinoma xenograft," *Clinical Cancer Research*, vol. 1, no. 11, pp. 1353–1358, 1995.

[52] H. R. Scott, D. C. McMillan, A. Crilly, C. S. McArdle, and R. Milroy, "The relationship between weight loss and interleukin 6 in non-small-cell lung cancer," *British Journal of Cancer*, vol. 73, no. 12, pp. 1560–1562, 1996.

[53] X. Zhou, J. L. Wang, J. Lu et al., "Reversal of cancer cachexia and muscle wasting by ActRIIB antagonism leads to prolonged survival," *Cell*, vol. 142, no. 4, pp. 531–543, 2010.

[54] J. M. Argiles and F. J. Lopez-Soriano, "The role of cytokines in cancer cachexia," *Medicinal Research Reviews*, vol. 19, no. 3, pp. 223–248, 1999.

[55] J. N. Gordon, S. R. Green, and P. M. Goggin, "Cancer cachexia," *Quarterly Journal of Medicine*, vol. 98, no. 11, pp. 779–788, 2005.

[56] M. J. Tisdale, "Tumor-host interactions," *Journal of Cellular Biochemistry*, vol. 93, no. 5, pp. 871–877, 2004.

[57] K. E. de Visser and L. M. Coussens, "The interplay between innate and adaptive immunity regulates cancer development," *Cancer Immunology, Immunotherapy*, vol. 54, no. 11, pp. 1143–1152, 2005.

[58] L. M. Coussens, W. W. Raymond, G. Bergers et al., "Inflammatory mast cells up-regulate angiogenesis during squamous epithelial carcinogenesis," *Genes & Development*, vol. 13, no. 11, pp. 1382–1397, 1999.

[59] H. N. Langstein and J. A. Norton, "Mechanisms of cancer cachexia," *Hematology/Oncology Clinics of North America*, vol. 5, no. 1, pp. 103–123, 1991.

[60] T. Watanabe, M. Shibata, H. Nishiyama et al., "Serum levels of rapid turnover porteins are decreased and related to systemic inflammation in patients with ovarian cancer," *Oncology Letters*, vol. 7, no. 2, pp. 373–377, 2013.

[61] L. Yang, L. M. DeBusk, K. Fukuda et al., "Expansion of myeloid immune suppressor Gr+CD11b+ cells in tumor-bearing host directly promotes tumor angiogenesis," *Cancer Cell*, vol. 6, no. 4, pp. 409–421, 2004.

[62] L. M. Coussens, C. L. Tinkle, D. Hanahan, and Z. Werb, "MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis," *Cell*, vol. 103, no. 3, pp. 481–490, 2000.

[63] R. D. Winfield, M. J. Delano, K. Pande, P. O. Scumpia, D. LaFace, and L. L. Moldawer, "Myeloid-derived suppressor cells in cancer cachexia syndrome: a new explanation for an old problem," *Journal of Parenteral and Enteral Nutrition*, vol. 32, no. 6, pp. 651–655, 2008.

[64] K. A. Baltgalvis, F. G. Berger, M. M. O. Pena, J. M. Davis, S. J. Muga, and J. A. Carson, "Interleukin-6 and cachexia in $APc^{Min/+}$ mice," *American Journal of Physiology: Regulatory Integrative and Comparative Physiology*, vol. 294, no. 2, pp. R393–R401, 2008.

[65] J. P. White, M. J. Puppa, S. Sato et al., "IL-6 regulation on skeletal muscle mitochondrial remodeling during cancer cachexia in the $APc^{Min/+}$ mouse," *Skeletal Muscle*, vol. 2, article 14, 2012.

[66] P. Todorov, P. Cariuk, T. McDevitt, B. Coles, K. Fearon, and M. Tisdale, "Characterization of a cancer cachectic factor," *Nature*, vol. 379, no. 6567, pp. 739–742, 1996.

[67] M. J. Lorite, M. G. Thompson, J. L. Drake, G. Carling, and M. J. Tisdale, "Mechanism of muscle protein degradation induced by a cancer cachectic factor," *British Journal of Cancer*, vol. 78, no. 7, pp. 850–856, 1998.

[68] C. L. Monitto, S.-M. Dong, J. Jen, and D. Sidransky, "Characterization of a human homologue of proteolysis-inducing factor and its role in cancer cachexia," *Clinical Cancer Research*, vol. 10, no. 17, pp. 5862–5869, 2004.

[69] R. Cabal-Manzano, P. Bhargava, A. Torres-Duarte, J. Marshall, P. Bhargava, and I. W. Wainer, "Proteolysis-inducing factor is expressed in tumours of patients with gastrointestinal cancers and correlates with weight loss," *British Journal of Cancer*, vol. 84, no. 12, pp. 1599–1601, 2001.

[70] M. J. Tisdale, "Are tumoral factors responsible for host tissue wasting in cancer cachexia?" *Future Oncology*, vol. 6, no. 4, pp. 503–513, 2010.

[71] A. S. Whitehouse and M. J. Tisdale, "Increased expression of the ubiquitin—proteasome pathway in murine myotubes by proteolysis-inducing factor (PIF) is associated with activation of the transcription factor NF-κB," *British Journal of Cancer*, vol. 89, no. 6, pp. 1116–1122, 2003.

[72] Y.-P. Li and M. B. Reid, "NF-κB mediates the protein loss induced by TNF-α in differentiated skeletal muscle myotubes," *American Journal of Physiology: Regulatory Integrative and Comparative Physiology*, vol. 279, no. 4, pp. R1165–R1170, 2000.

[73] N. Mizushima, A. Yamamoto, M. Matsui, T. Yoshimori, and Y. Ohsumi, "In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker," *Molecular Biology of the Cell*, vol. 15, no. 3, pp. 1101–1111, 2004.

[74] F. Penna, D. Costamagna, F. Pin et al., "Autophagic degradation contributes to muscle wasting in cancer cachexia," *The American Journal of Pathology*, vol. 182, no. 4, pp. 1367–1378, 2013.

[75] S. A. Reed, P. B. Sandesara, S. M. Senf, and A. R. Judge, "Inhibition of FoxO transcriptional activity prevents muscle fiber atrophy during cachexia and induces hypertrophy," *The FASEB Journal*, vol. 26, no. 3, pp. 987–1000, 2012.

[76] S. Acharyya, K. J. Ladner, L. L. Nelsen et al., "Cancer cachexia is regulated by selective targeting of skeletal muscle gene products," *The Journal of Clinical Investigation*, vol. 114, no. 3, pp. 370–378, 2004.

[77] A. Bonetto, T. Aydogdu, N. Kunzevitzky et al., "STAT3 activation in skeletal muscle links muscle wasting and the acute phase response in cancer cachexia," *PLoS ONE*, vol. 6, no. 7, Article ID e22538, 2011.

[78] T. M. Watchorn, I. Waddell, N. Dowidar, and J. A. Ross, "Proteolysis-inducing factor regulates hepatic gene expression via the transcription factors NF-κB and STAT3," *The FASEB Journal*, vol. 15, no. 3, pp. 562–564, 2001.

[79] F. Penna, D. Costamagna, A. Fanzani, G. Bonelli, F. M. Baccino, and P. Costelli, "Muscle wasting and impaired myogenesis in tumor bearing mice are prevented by ERK inhibition," *PLoS ONE*, vol. 5, no. 10, Article ID e13604, 2010.

[80] W. A. He, E. Berardi, V. M. Cardillo et al., "NF-κB-mediated Pax7 dysregulation in the muscle microenvironment promotes cancer cachexia," *The Journal of Clinical Investigation*, vol. 123, no. 11, pp. 4821–4835, 2013.

[81] F. Relaix, X.-J. Wei, X. Wu, and D. A. Sassoon, "*Peg3/Pw1* is an imprinted gene involved in the TNF-NFκB signal transduction pathway," *Nature Genetics*, vol. 18, no. 3, pp. 287–291, 1998.

[82] D. Coletti, E. Yang, G. Marazzi, and D. Sassoon, "TNFα inhibits skeletal myogenesis through a PW1-dependent pathway by recruitment of caspase pathways," *The EMBO Journal*, vol. 21, no. 4, pp. 631–642, 2002.

[83] C. M. Op den Kamp, R. C. Langen, F. J. Snepvangers et al., "Nuclear transcription factor κ B activation and protein turnover adaptations in skeletal muscle of patients with progressive stages of lung cancer cachexia," *The American Journal of Clinical Nutrition*, vol. 98, no. 3, pp. 738–748, 2013.

[84] D. Cai, J. D. Frantz, N. E. Tawa Jr. et al., "IKKβ/NF-κB activation causes severe muscle wasting in mice," *Cell*, vol. 119, no. 2, pp. 285–298, 2004.

[85] A. MacCiò, C. Madeddu, G. Gramignano et al., "A randomized phase III clinical trial of a combined treatment for cachexia in patients with gynecological cancers: evaluating the impact on metabolic and inflammatory profiles and quality of life," *Gynecologic Oncology*, vol. 124, no. 3, pp. 417–425, 2012.

[86] C. Wu, S. A. Fernandez, T. Criswell et al., "Disrupting cytokine signaling in pancreatic cancer: a phase I/II study of etanercept in combination with gemcitabine in patients with advanced disease," *Pancreas*, vol. 42, no. 5, pp. 813–818, 2013.

[87] G. Mantovani, A. MacCiò, C. Madeddu et al., "Phase II nonrandomized study of the efficacy and safety of COX-2 inhibitor celecoxib on patients with cancer cachexia," *Journal of Molecular Medicine*, vol. 88, no. 1, pp. 85–92, 2010.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 560629, 9 pages
http://dx.doi.org/10.1155/2014/560629

Review Article

Macrophage Plasticity in Skeletal Muscle Repair

Elena Rigamonti,[1] Paola Zordan,[1] Clara Sciorati,[1] Patrizia Rovere-Querini,[1,2] and Silvia Brunelli[1,3]

[1] *Division of Regenerative Medicine, Stem Cells and Gene Therapy, San Raffaele Scientific Institute, Via Olgettina 58, 20132 Milano, Italy*
[2] *San Raffaele University, Via Olgettina 58, 20132 Milano, Italy*
[3] *Department of Health Sciences, University of Milano-Bicocca, via Cadore 48, 20900 Monza, Italy*

Correspondence should be addressed to Silvia Brunelli; silvia.brunelli@unimib.it

Received 18 January 2014; Revised 13 March 2014; Accepted 31 March 2014; Published 17 April 2014

Academic Editor: Pura Muñoz-Cánoves

Macrophages are one of the first barriers of host defence against pathogens. Beyond their role in innate immunity, macrophages play increasingly defined roles in orchestrating the healing of various injured tissues. Perturbations of macrophage function and/or activation may result in impaired regeneration and fibrosis deposition as described in several chronic pathological diseases. Heterogeneity and plasticity have been demonstrated to be hallmarks of macrophages. In response to environmental cues they display a proinflammatory (M1) or an alternative anti-inflammatory (M2) phenotype. A lot of evidence demonstrated that after acute injury M1 macrophages infiltrate early to promote the clearance of necrotic debris, whereas M2 macrophages appear later to sustain tissue healing. Whether the sequential presence of two different macrophage populations results from a dynamic shift in macrophage polarization or from the recruitment of new circulating monocytes is a subject of ongoing debate. In this paper, we discuss the current available information about the role that different phenotypes of macrophages plays after injury and during the remodelling phase in different tissue types, with particular attention to the skeletal muscle.

1. Role of Macrophages in Inflammation Resolution and Tissue Remodelling

Macrophages are essential for the efficient healing of numerous tissues. They contribute to homeostatic tissue remodelling during foetal life [1, 2] and in several tissues in the adult. The healing process consists of overlapping phases of inflammation, tissue formation, and remodelling with reorganization of vasculature and extracellular matrix. Macrophages participate in all the different phases of tissue repair: they can promote phagocytosis of cellular debris and apoptotic neutrophils and produce cytokines that may help orchestrate the healing response. However, due to the release of proinflammatory cytokines and cytotoxic radical species, uncontrolled activity of macrophages may also be detrimental to tissue repair. Indeed, several human diseases are characterized by attenuated repair responses and imbalances in the inflammatory response with increased number of infiltrating macrophages [3–5]. Heterogeneity and plasticity of macrophages could explain these apparently contrasting roles in tissue healing. All macrophages express common markers such as CD11b (Mac1 or CR3), CD68, and CD115 (M-CSF receptor). However, at least two distinct macrophage populations have been identified: the classically activated M1 phenotype and the alternative activated M2 phenotype [6]. Classically activated M1 macrophages are induced *in vitro* by IFNγ, alone or in concert with microbial stimuli (e.g., LPS) or selected cytokines (e.g., TNF and GM-CSF). They have proinflammatory functions: they produce effector molecules (reactive oxygen and nitrogen intermediates) and inflammatory cytokines (IL-1β, TNFα, and IL-6) and participate as inducer and effector cells in polarized Th1 responses. Alternatively activated M2 macrophages comprise cells exposed to low concentrations of M-CSF in the presence of IL-4, IL-13, or IL-10. They participate in polarized Th2 reactions, parasite clearance, damping of inflammation, and promotion

of angiogenesis and tissue remodelling [7, 8]. *In vivo*, the identification of macrophage phenotype is complicated since macrophages are obviously exposed to a microenvironment that is more complex respect to cell culture conditions, and they display characteristics that do not conform to the *in vitro* defined phenotypic categories. Therefore, the *in vivo* classification of macrophages in two polarized states (M1 versus M2) sounds as an oversimplification. Therefore, in the last years characterization of macrophage phenotype *in vivo* during tissue repair has been a matter of active investigation. Macrophage activation has been described as a dynamic process: the same cell may initially induce proinflammatory and cytotoxic reactions and later may take part in the resolution of inflammation and wound healing [9]. A common scenario is emerging, in which soon after injury infiltrating macrophages are mainly proinflammatory M1 macrophages, whereas M2 macrophages are the primary effectors of later stages of tissue repair or remodelling phases [10–12]. Recent evidence has also shed light on the functional cross-talk between macrophages and stem/progenitor cells, which may contribute to repair and remodelling in different tissue/organs [13–15].

Specific examples of the origin and functions of macrophages during healing of various tissues are illustrated below, with particular emphasis on skeletal muscle.

Macrophages play a crucial role in the restoration of skin integrity and homeostasis and exert distinct functions during the multiple phases of skin repair, despite the underlying molecular mechanisms remaining partially unclear [16, 17]. Transgenic mice that express the human diphtheria toxin receptor (DTR) under the control of the CD11b promoter have been shown to allow a conditional depletion of macrophages [18]. Using these transgenic mice, Mirza et al. demonstrated that macrophage depletion during wound healing leads to delayed reepithelialization, reduced collagen deposition, impaired angiogenesis, and finally wound closure [17]. Interestingly, Lucas et al. showed that during the early phases of skin repair, infiltrating macrophages are alternatively activated and express high levels of growth factors, VEGFα and TGFβ, which contribute, respectively, to wound angiogenesis and myofibroblast differentiation [16]. During the midstage of the skin repair response, macrophages still express VEGFα and TGFβ but to a lesser extent and they are crucial for vessels stabilization and scar formation. More recently, TGFβ has been described to regulate wound healing through TLR4 receptor. Indeed, TLR4$^{-/-}$ mice display impaired skin wound healing with decreased macrophage infiltration and reduced levels of TGFβ [19].

Dynamic changes in monocyte/macrophage phenotype have been described also in a model of myocardial injury. Macrophages have been suggested to be beneficial for myocardial wound healing. Optimum outcome of myocardial injury is strictly related to the balance between debris clearance and myocardial extracellular matrix repair. Liposome-mediated depletion of infiltrating macrophages after myocardial injury results in persistence of cellular debris, impaired vascularization, and myofibroblast infiltration and ultimately leads to ineffective scar formation. After injury, macrophage-depleted mice display cardiac complications and ultimately a significant decreased survival [20]. Two different kinds of monocyte/macrophages populations have been suggested to infiltrate the heart after injury: Ly-6C^{high} proinflammatory monocytes firstly arrive via CCR2 receptor and scavenge necrotic debris; subsequently Ly-6C^{low} preferentially accumulate and promote an anti-inflammatory response and granulation tissue formation [21]. Similar kinetics of monocyte infiltration has been observed also in patients with acute myocardial infarction [22]. Moreover, microarray analyses on RNA of macrophages isolated from infarcted tissues confirmed the expression of proinflammatory (M1) markers in the tissue early after injury and of alternative activated (M2) macrophage markers later during scar tissue formation [23].

The plasticity of macrophages has been reported to play a role also in parenchymal organ diseases, such as liver or lung fibrosis. Liver fibrosis is a common consequence of chronic liver disease and current evidence suggests that this process is mainly driven by a local inflammatory response [24, 25]. Experimental models of liver fibrosis highlight the importance of hepatic resident macrophages, the Kupffer cells, for sustaining inflammation as well as activating the hepatic stem cells (HSC) [26]. However, fibrosis largely depends on recruitment of monocytes into the liver [18, 27]. In a reversible model of liver fibrosis two functionally distinct types of macrophages have been demonstrated to regulate the outcome of the fibrotic response [18]: during the injury phase, infiltrating macrophages promote myofibroblast proliferation and matrix deposition by secreting high amounts of TGFβ and TNFα, whereas during the recovery phase they sustain matrix degradation, probably by releasing MMP13 [28]. Profibrogenic macrophages have been shown to derive mainly from circulating Ly-6C^{high} proinflammatory monocytes, which massively invade injured liver via the CCR2 receptor both in mice and in humans [29–31]. Similarly, a critical role of macrophages in regulating lung fibrosis has been recently described. Evidence supports the involvement of alternative activated macrophages (M2) in lung fibrotic response via secretion of TGFβ [32]. These results were corroborated by recent observational studies in humans which highlight the presence of M2 macrophages markers in lung diseases: CD163, CCL18, CCL22, and CD206 [33, 34].

2. A Case for Macrophages in Acute and Chronic Muscle Damages

The plasticity of macrophages in response to environmental cues has been largely investigated in the skeletal muscle [35–37]. Muscle inflammation is a common physiologic response to exercise and a typical feature of acute and chronic muscle damages. Muscle regeneration and healing after damage mainly depend upon quiescent muscle stem cells, the satellite cells, localized between the basal lamina and the muscle fiber membrane [38]. Upon muscle injury, satellite cells activate, start proliferating, and, subsequently, differentiating into new myotubes that replace damaged muscle [39, 40]. Beside

satellite cells, the inflammatory cells that infiltrate the injured muscle deeply influence the outcome of muscle regeneration.

2.1. Acute Muscle Injury and Macrophage Activation. Skeletal muscle sterile injury triggers a potent inflammatory response characterized by a rapid and sequential invasion of leukocyte populations that persist during muscle repair, regeneration, and growth. The regeneration process includes an initial proinflammatory phase characterized by release of cytokines and chemokines which promote infiltration of immune cells to the site of damage in order to remove cellular debris [41].

Neutrophils are the first leukocyte population in damaged tissue. They appear within 2 h of muscle damage, reaching a maximum in concentration between 6 and 24 h postinjury and then rapidly decreasing. The actual role of neutrophils in damaged skeletal muscle is still debated. They release molecules (proteolytic enzymes, oxygen-derived reactive species) that may contribute to muscle membrane lysis and, therefore, to damage extension [42]. However, neutrophils have also been suggested to facilitate muscle regeneration by removing tissue debris from the injured area as well as by activating satellite cells [43]. Recent results indicated that the supportive and/or deleterious effects of neutrophils on skeletal muscle might rely on the degree of their activation. Indeed, during modified mechanical loading, neutrophils are efficiently eliminated with no significant muscle fiber injury. Conversely, the presence of microbial products leads to significant neutrophil infiltration and muscle fiber damage [44].

Shortly after neutrophil invasion, macrophages begin to accumulate and, subsequently, become the dominant leukocyte population [45, 46]. They are mainly derived from blood monocytes that have crossed the vessel endothelial barrier to reach the tissue [47]. Macrophages are professional scavengers of apoptotic cells and debris and produce a pattern of signals involved in myogenic precursors activation, matrix remodelling, and neovessel formation [48, 49]. *In vivo* studies have unequivocally shown that macrophages play a pivotal role in the muscle repair process [15, 50–54]. Indeed, data from several models of muscle injury (hindlimb ischemia, freeze-injury, unloading/reloading sequences, and myotoxic agent injections) indicate that impairment of macrophage recruitment in injured muscle results in delayed tissue regeneration in terms of appearance of regenerating centronucleated myofibers and persistence of intramuscular adipocytes and fibrosis [55]. More recently, other cell types, including eosinophils and fibroadipogenic precursors, have been shown to contribute to the rapid clearance of necrotic debris and, subsequently, proper muscle regeneration [56].

During the early stages of acute muscle injury, infiltrating and muscle-resident macrophages associated with the epimisyal and perimysial connective tissue contribute in locally attracting monocytes from the blood by secreting chemokines, such as MCP1/CCL2 [57]. Indeed, the expression of MCP1/CCL2 receptor (CCR2) on bone marrow derived cells is critical for normal skeletal muscle regeneration. Mice defective for CCR2 ($CCR2^{-/-}$) display severe impairments in macrophage recruitment and skeletal muscle regeneration following cardiotoxin (CTX)-induced injury [58]. Interestingly, $MCP1^{-/-}$ mice exhibit an intermediate phenotype compared with $CCR2^{-/-}$ mice in terms of macrophage recruitment to the site of injury, resolution of necrosis, and muscle regeneration, thus suggesting that other chemokines, in addition to MCP1, may activate CCR2-dependent regenerative processes [59]. Similarly, CXCL16 has also been shown to regulate monocyte/macrophage entry into the injured muscle [60]. Genetic disruption of CXCL16 pathway resulted in defective homing of macrophages and persistent infiltration of neutrophils, leading to sustained inflammation, impaired muscle regeneration, and scar deposition.

Two different macrophage populations have been described in injured/regenerating skeletal muscle. Arnold et al. [61] identified a population of circulating monocytes, which are selectively recruited to the site of damage and display a proinflammatory phenotype. They secrete inflammatory signals, including TNFα, IL-1β, and MCP1, and dispose of fiber remnants. Moreover, macrophages infiltrating damaged muscle have been recently shown to express inducible nitric oxide synthase (iNOS), a typical marker of M1 macrophages [62]. The phagocytosis of either apoptotic or necrotic myogenic cells apparently sustains the functional polarization of macrophages towards an anti-inflammatory phenotype. M2 macrophages contribute to dampen the inflammatory response by secreting TGFβ and IL-10. Moreover, they sustain fiber reconstitution by secreting cytokines that may play a trophic function, such as IGF1 and IL-10. In particular, IL-10 is mainly produced by infiltrating macrophages and its secretion is necessary to sustain viability and allow differentiation and fusion of the myogenic progenitor mesoangioblasts into terminally differentiated myofibers [15]. The sequential presence of proinflammatory and then anti-inflammatory macrophages has been also demonstrated in human muscles. Both subsets of macrophages have been identified in injured/regenerating human muscles. Macrophages expressing M1 markers preferentially associate with proliferating satellite cells, whereas at the time of myogenic differentiation macrophages mainly express anti-inflammatory M2 markers [63].

The cellular and molecular pathways involved in the regulation of macrophage phenotype transition during muscle injury/regeneration have been deeply investigated in the latest years. The cAMP response element-binding protein (CREB) has been demonstrated to be a crucial transcription factor for the upregulation of M2-associated gene while repressing M1 activation. Deletion of two CREB binding sites from the C/EBPβ gene promoter blocks the downstream induction of anti-inflammatory genes associated with M2-like macrophage activation, whereas the inflammatory (M1) genes are not affected. Upon muscle injury, mice carrying the mutated C/EBPβ promoter efficiently clear injured muscle from necrotic debris but display severe defects in muscle fiber regeneration, thus confirming that the persistence of inflammatory macrophages in damaged muscle of these mice is not sufficient for effective regeneration [64]. Another molecule playing a key role in regulating macrophage phenotypic

transition and muscle recovery is the MAP kinase phosphatase (MKP)-1 [65]. Gene-expression analyses on sorted MKP-$1^{-/-}$ muscle macrophages indicated that MKP-1 controls the inflammatory response as well as the switch from early pro- to late anti-inflammatory macrophage phenotype via p38 MAPK downregulation. Mice deficient in MKP-1 display defective muscle regeneration with persistence of damage and impaired growth of regenerating myofibers. Interestingly, this phenotype could be completely restored by MKP-$1^{+/+}$ bone-marrow transplantation, strongly suggesting dispensability of this protein for satellite cell-dependent myofiber repair [65]. Recently, AMP-activated protein kinase (AMPK)-α1 has also been demonstrated to play a significant role in the regulation of macrophage skewing during skeletal muscle regeneration. Increase in AMPK activity has been associated with a decreased proinflammatory status of macrophages [66]. Indeed AMPK$\alpha 1^{-/-}$ macrophages fail to adopt an anti-inflammatory (M2) phenotype and display a defect in the phagocytic activity [67]. Consistently, mice bearing a specific deletion of AMPKα1 in myeloid-cells show a significant delay in skeletal muscle regeneration paralleled by a decreased number of M2 macrophages [67]. More recently, a population of regulatory T cell (Treg) has been shown to infiltrate injured muscles and support muscle repair by modulating the several steps of the regeneration process. Interestingly, muscle Treg cells promote the switch between pro- and anti-inflammatory macrophages; the precise mechanisms and the potentially responsible molecules are currently under investigation [68].

2.2. Chronic Muscle Injury. The study of the molecular mechanisms underlying the role of macrophage subpopulations in muscle repair after acute muscle injury could blaze new trails in the comprehension of onset and progression of chronic muscle diseases, even if in these conditions macrophages may exert a more complex role, in response to a more complex and heterogeneous scenario.

In genetic diseases of the muscle, such as the *muscle dystrophies*, the noxa cannot be eliminated. The genetic defect usually affects the structure of the muscle fiber: membranes become more fragile, leading to necrosis [69]. Since the stem cell compartment undergoes a progressive depletion/exhaustion and necrosis does not abate, the tissue architecture is progressively disrupted [70]. In addition, the release of adjuvant stimuli, that activate the innate and acquired immune responses, and the generation of reactive oxygen and nitrogen species, may impinge on macrophage survival/polarization and function [54, 68, 71].

Several mouse models of chronic muscle damage exist and allow a better understanding of the role of macrophage plasticity during the onset and progression of diseases. Moreover they are essential in developing a new pharmacological or stem cell based clinical strategy.

In the *mdx* mice, a model for Duchenne muscle dystrophy, the early stage of the disease is characterized by an innate immunity response that is similar to that occurring after an acute injury, with a massive invasion of neutrophils and M1-like macrophages. The classical activation of M1 macrophages is driven by proinflammatory Th1 cytokines, especially TNFα and IFNγ. Both cytokines are highly expressed in *mdx* muscles and they possibly promote muscle damage during the acute stage of the pathology [37, 72]. Antibody and pharmacological blockade of TNFα in young *mdx* mice results in a delayed and significantly reduced amount of skeletal muscle damage [73, 74]. IFNγ stimulation of macrophages isolated from *mdx* muscles significantly increases muscle cell lysis *in vitro* [72]. However, *in vivo* ablation of IFNγ in young *mdx* mice does not affect muscle fiber damage and only partially reduces iNOS expression without decreasing macrophage cytotoxicity [75]. Classically activated M1 macrophages persist in the dystrophic muscle due to the unremitting inflammatory response and induce further muscle damage through the production of cytotoxic levels of nitric oxide (NO) by iNOS [72].

The role of NO in the muscle is nevertheless more complex. The lack of dystrophin [76, 77] disrupts indeed the recruitment of another nitric oxide synthase isoform, the neuronal NOS (nNOS), to the sarcolemma, thus affecting NO production in muscle fibers [78, 79] and contributing to the severity of the dystrophic phenotype [80, 81]. The rescue of function in *mdx* or dystrophin/utrophin double-knockout mice by overexpressing an nNOS transgene has demonstrated that NO controls disease progression and corrects the balance in macrophage subpopulations [82, 83]. In dystrophic mice the early M1 invasion is indeed followed by the recruitment of a subpopulation of M2 macrophages, expressing CD206, IL-10, and Arginase, that are referred to as M2a; these cells reduce NO mediated cytotoxicity of M1 macrophages by competing for the substrate arginine [75, 84]. Subsequent invasion of the dystrophic muscle by another subpopulation of alternatively activated macrophages, defined as M2c and expressing CD163, further contributes to M1 deactivation and is associated with tissue healing and progression to the regenerative phase [72, 84]. The persistence of inflammation at later stages promotes excessive connective tissue deposition that leads to muscle fibrosis, characteristic of dystrophy [84].

In the presence of the nNOS transgene a decrease in M2c macrophages in the muscle of dystrophic mice was observed, paralleled by a significant reduction of fibrosis. The nNOS transgene has no effect on the concentration of cytolytic M1 macrophages [83].

The role of NO in modulating the inflammatory response in the dystrophic muscle has been demonstrated by treating another mouse model of dystrophy, the alpha-SG KO mouse, with the NO donor Molsidomine [85, 86]. Molsidomine administration leads to a reduction of the inflammatory infiltrate, in particular in terms of number of neutrophils and classically activated macrophages. In addition, most of remaining macrophages coexpress both markers of classical and alternative activation (CD206+ CD163+ CD86+) and might represent a transitional population, which maintains the ability to sustain the proliferation and differentiation of myogenic precursors without contributing to the deposition of collagen and persistence of fibrosis [8, 37].

Inflammatory myopathies are another class of chronic muscle diseases. They are heterogeneous and classically comprise *polymyositis* [52], *dermatomyositis* (DM), and *sporadic*

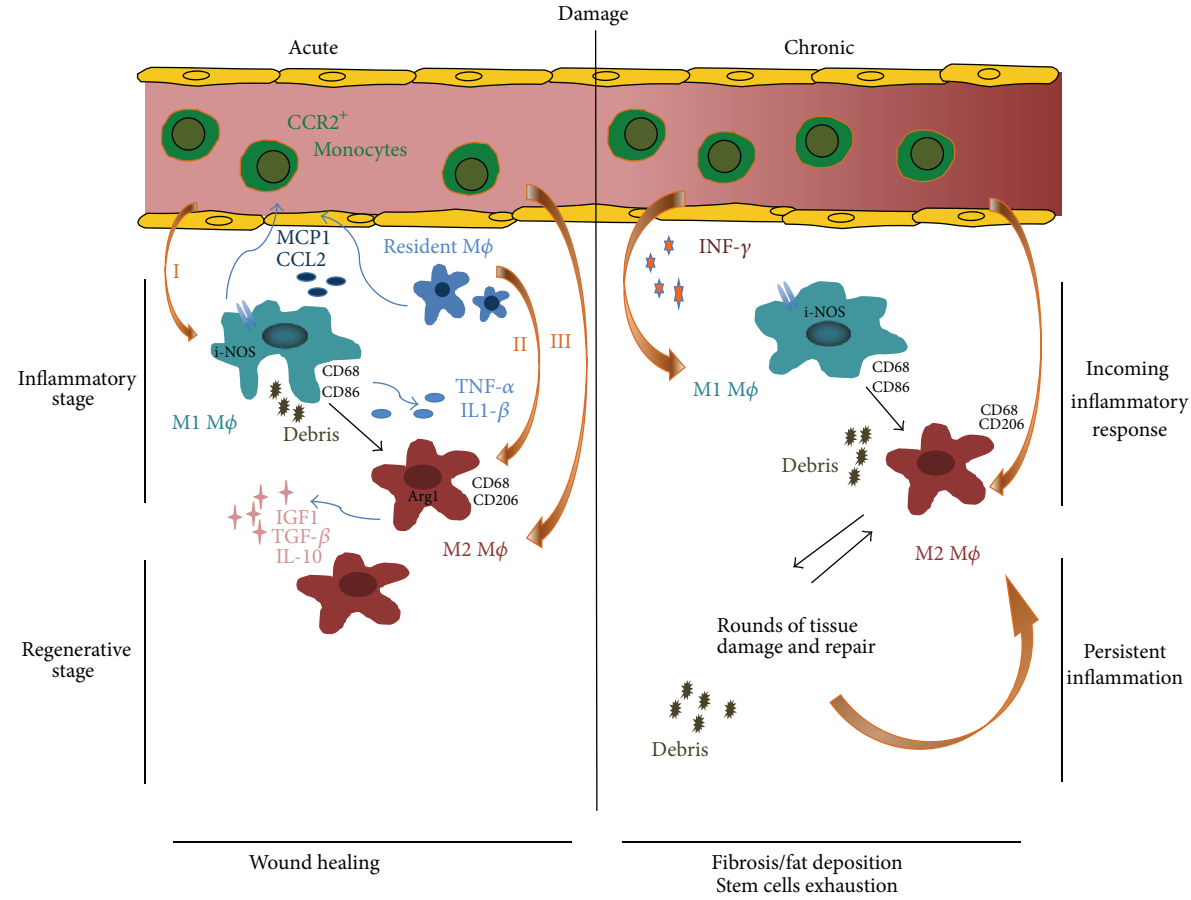


FIGURE 1: Macrophages in acute and chronic muscle damage. The innate immune system through M1 macrophages activates an inflammatory response: secretion of cytokines triggers the clearance of the tissue from the debris and the activation of stem cells. Phagocytosis of apoptotic and necrotic cells induces an M1 to M2 macrophage transition (I). M2 polarized macrophages originate from resident macrophages (II) or can be recruited from circulating monocyte (III). This is a regenerative stage during which stem cells differentiate and the damage is resolved. In chronic diseases several rounds of damage and repair occur: both M1 and M2 polarized macrophages coexist in the tissue, recruited from monocytes. This persistent inflammation leads to fibrosis, fat deposition, and exhaustion of the stem cell pool.

inclusion body myositis (IBM) [87]. Despite these disorders differing in prognosis and response to treatment, common clinical signs are muscle mononuclear cells infiltration and myofiber degeneration [88]. Important immunological features include also autoantibodies and autoreactive T lymphocytes with the overexpression of major histocompatibility complex class I molecules on the surface of fibers [89]. In DM the humeral immunity due to $CD4^+$ cells and B cells plays a predominant role, while PM and IBM disorders are mediated by cytotoxic $CD8^+$ T cells which attack skeletal muscle fibers [88, 90]. Interestingly, macrophage infiltration is common in all inflammatory myopathies. At present, few data are available concerning the phenotype and the role of macrophages in the pathology of inflammatory myopathies. Analyses of muscle biopsies demonstrated that in areas of severe inflammation and necrosis, macrophages express both proinflammatory and anti-inflammatory markers. Indeed, in PM, macrophages are highly positive for iNOS and TGFβ, thus suggesting the existence of two possible macrophage subpopulations, which could modulate the inflammatory response [91]. Moreover, Reimann et al. demonstrated that the macrophage migration inhibitory factor (MIF) is highly expressed in muscle samples of human PM. MIF is a T cell and macrophage derived proinflammatory cytokine with antiapoptotic, proproliferative, and chemotactic effects. In muscle biopsies of PM, MIF has been detected not only in inflammatory cells but also on muscle fiber membrane, thus suggesting a potential role of MIF in the onset of the disease [92]. In addition to the classical PM, DM, and sporadic IBM, *immune-mediated necrotizing myopathy* (IMNM) is another important class of immune-mediated myopathies [93]. More recently, it has been defined as a Th1-M1-mediated disease due to high levels of proinflammatory cytokines IFNγ, TNFα, and IL-12 that have been detected in biopsy specimens; by contrast no difference was observed for markers of alternative activation of macrophages between patients and healthy control biopsies [94]. Further investigations are required to better characterize the molecular mechanism of the immune response in inflammatory myopathies and ultimately to design potential therapeutic approaches.

3. Conclusions

Research in the past few years has highlighted a pivotal role of macrophages in tissue repair and remodelling. Macrophages are renowned for their plasticity and heterogeneity, which have been described not only *in vitro* but also in various physiological and pathological contests. Evidence indicated that macrophages are extremely versatile cells that can undergo phenotype changes according to specific environmental cues. In skeletal muscle, after acute injury, proinflammatory M1 macrophages firstly arrive to clear debris and are sequentially replaced by healing M2 macrophages that sustain tissue repair and regeneration. In chronic muscle injury, both M1 and M2 macrophages coexist but fail to promote tissue repair and homeostasis recovery (Figure 1). The efforts of the next years are likely to identify the molecular determinants of macrophage polarization in order to possibly develop effective targeted therapies for genetic defects of the tissue and muscle diseases associated with chronic inflammation.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

This work was supported by the European Community's framework programme FP7/2007–2013 under Grant Agreements no. 241440 (ENDOSTEM) (to Silvia Brunelli and Patrizia Rovere-Querini), the Italian Ministry of Health (Fondo per gli Investimenti della Ricerca di Base-IDEAS to Patrizia Rovere-Querini and Ricerca Finalizzata to Patrizia Rovere-Querini), and the Italian Ministry of Research and University (PRIN 2010-11, 20108YB5W3_007 to Silvia Brunelli).

References

[1] J. Y. Bertrand, A. Jalil, M. Klaine, S. Jung, A. Cumano, and I. Godin, "Three pathways to mature macrophages in the early mouse yolk sac," *Blood*, vol. 106, no. 9, pp. 3004–3011, 2005.

[2] D. A. Ovchinnikov, "Macrophages in the embryo and beyond: much more than just giant phagocytes," *Genesis*, vol. 46, no. 9, pp. 447–462, 2008.

[3] A. Sica, P. Invernizzi, and A. Mantovani, "Macrophage plasticity and polarization in liver homeostasis and pathology," *Hepatology*, 2013.

[4] A. Mantovani and M. Locati, "Tumor-associated macrophages as a paradigm of macrophage plasticity, diversity, and polarization: lessons and open questions," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 33, no. 7, pp. 1478–1483, 2013.

[5] G. P. Fadini, R. Cappellari, M. Mazzucato, C. Agostini, S. V. de Kreutzenberg, and A. Avogaro, "Monocyte-macrophage polarization balance in pre-diabetic individuals," *Acta Diabetologica*, vol. 50, no. 6, pp. 977–982, 2013.

[6] A. Mantovani, A. Sica, and M. Locati, "Macrophage polarization comes of age," *Immunity*, vol. 23, no. 4, pp. 344–346, 2005.

[7] A. Sica and A. Mantovani, "Macrophage plasticity and polarization: in vivo veritas," *Journal of Clinical Investigation*, vol. 122, no. 3, pp. 787–795, 2012.

[8] D. M. Mosser and J. P. Edwards, "Exploring the full spectrum of macrophage activation," *Nature Reviews Immunology*, vol. 8, no. 12, pp. 958–969, 2008.

[9] A. Mantovani, S. K. Biswas, M. R. Galdiero, A. Sica, and M. Locati, "Macrophage plasticity and polarization in tissue repair and remodelling," *The Journal of Pathology*, vol. 229, no. 2, pp. 176–185, 2013.

[10] J. M. Daley, S. K. Brancato, A. A. Thomay, J. S. Reichner, and J. E. Albina, "The phenotype of murine wound macrophages," *Journal of Leukocyte Biology*, vol. 87, no. 1, pp. 59–67, 2010.

[11] S. K. Brancato and J. E. Albina, "Wound macrophages as key regulators of repair: origin, phenotype, and function," *American Journal of Pathology*, vol. 178, no. 1, pp. 19–25, 2011.

[12] A. Sindrilaru, T. Peters, S. Wieschalka et al., "An unrestrained proinflammatory M1 macrophage population induced by iron impairs wound healing in humans and mice," *Journal of Clinical Investigation*, vol. 121, no. 3, pp. 985–997, 2011.

[13] P. F. Lesault, M. Théret, M. Magnan et al., "Macrophages improve survival, proliferation and migration of engrafted myogenic precursor cells into MDX skeletal muscle," *PLoS ONE*, vol. 7, no. 10, Article ID e46698, 2012.

[14] O. Gonzalez-Perez, F. Gutierrez-Fernandez, V. Lopez-Virgen, J. Collas-Aguilar, A. Quinones-Hinojosa, and J. M. Garcia-Verdugo, "Immunological regulation of neurogenic niches in the adult brain," *Neuroscience*, vol. 226, pp. 270–281, 2012.

[15] L. Bosurgi, G. Corna, M. Vezzoli et al., "Transplanted mesoangioblasts require macrophage IL-10 for survival in a mouse model of muscle injury," *Journal of Immunology*, vol. 188, no. 12, pp. 6267–6277, 2012.

[16] T. Lucas, A. Waisman, R. Ranjan et al., "Differential roles of macrophages in diverse phases of skin repair," *Journal of Immunology*, vol. 184, no. 7, pp. 3964–3977, 2010.

[17] R. Mirza, L. A. DiPietro, and T. J. Koh, "Selective and specific macrophage ablation is detrimental to wound healing in mice," *American Journal of Pathology*, vol. 175, no. 6, pp. 2454–2462, 2009.

[18] J. S. Duffield, S. J. Forbes, C. M. Constandinou et al., "Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair," *Journal of Clinical Investigation*, vol. 115, no. 1, pp. 56–65, 2005.

[19] H. Suga, M. Sugaya, H. Fujita et al., "TLR_4, rather than TLR_2, regulates wound healing through TGF-beta and CCL_5 expression," *Journal of Dermatological Science*, vol. 73, no. 2, pp. 117–124, 2014.

[20] M. J. van Amerongen, M. C. Harmsen, N. van Rooijen, A. H. Petersen, and M. J. A. van Luyn, "Macrophage depletion impairs wound healing and increases left ventricular remodeling after myocardial injury in mice," *American Journal of Pathology*, vol. 170, no. 3, pp. 818–829, 2007.

[21] M. Nahrendorf, F. K. Swirski, E. Aikawa et al., "The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions," *Journal of Experimental Medicine*, vol. 204, no. 12, pp. 3037–3047, 2007.

[22] H. Tsujioka, T. Imanishi, H. Ikejima et al., "Impact of heterogeneity of human peripheral blood monocyte subsets on myocardial salvage in patients with primary acute myocardial infarction," *Journal of the American College of Cardiology*, vol. 54, no. 2, pp. 130–138, 2009.

[23] C. Troidl, H. Möllmann, H. Nef et al., "Classically and alternatively activated macrophages contribute to tissue remodelling after myocardial infarction," *Journal of Cellular and Molecular Medicine*, vol. 13, no. 9, pp. 3485–3496, 2009.

[24] R. Safadi, M. Ohta, C. E. Alvarez et al., "Immune stimulation of hepatic fibrogenesis by CD8 cells and attenuation by transgenic interleukin-10 from hepatocytes," *Gastroenterology*, vol. 127, no. 3, pp. 870–882, 2004.

[25] O. Park, W.-I. Jeong, L. Wang et al., "Diverse roles of invariant natural killer T cells in liver injury and fibrosis induced by carbon tetrachloride," *Hepatology*, vol. 49, no. 5, pp. 1683–1694, 2009.

[26] C. A. Rivera, B. U. Bradford, K. J. Hunt et al., "Attenuation of CCl_4-induced hepatic fibrosis by $GdCl_3$ treatment or dietary glycine," *American Journal of Physiology: Gastrointestinal and Liver Physiology*, vol. 281, no. 1, pp. G200–G207, 2001.

[27] M. Imamura, T. Ogawa, Y. Sasaguri, K. Chayama, and H. Ueno, "Suppression of macrophage infiltration inhibits activation of hepatic stellate cells and liver fibrogenesis in rats," *Gastroenterology*, vol. 128, no. 1, pp. 138–146, 2005.

[28] J. A. Fallowfield, M. Mizuno, T. J. Kendall et al., "Scar-associated macrophages are a major source of hepatic matrix metalloproteinase-13 and facilitate the resolution of murine hepatic fibrosis," *Journal of Immunology*, vol. 178, no. 8, pp. 5288–5295, 2007.

[29] E. Liaskou, H. W. Zimmermann, K. K. Li et al., "Monocyte subsets in human liver disease show distinct phenotypic and functional characteristics," *Hepatology*, vol. 57, no. 1, pp. 385–398, 2013.

[30] K. R. Karlmark, R. Weiskirchen, H. W. Zimmermann et al., "Hepatic recruitment of the inflammatory $Gr1^+$ monocyte subset upon liver injury promotes hepatic fibrosis," *Hepatology*, vol. 50, no. 1, pp. 261–274, 2009.

[31] C. Mitchell, D. Couton, J.-P. Couty et al., "Dual role of CCR_2 in the constitution and the resolution of liver fibrosis in mice," *American Journal of Pathology*, vol. 174, no. 5, pp. 1766–1775, 2009.

[32] M. A. Gibbons, A. C. MacKinnon, P. Ramachandran et al., "Ly6Chi monocytes direct alternatively activated profibrotic macrophage regulation of lung fibrosis," *American Journal of Respiratory and Critical Care Medicine*, vol. 184, no. 5, pp. 569–581, 2011.

[33] D. V. Pechkovsky, A. Prasse, F. Kollert et al., "Alternatively activated alveolar macrophages in pulmonary fibrosis-mediator production and intracellular signal transduction," *Clinical Immunology*, vol. 137, no. 1, pp. 89–101, 2010.

[34] E. Bargagli, A. Prasse, C. Olivieri, J. Muller-Quernheim, and P. Rottoli, "Macrophage-derived biomarkers of idiopathic pulmonary fibrosis," *Pulmonary Medicine*, vol. 2011, Article ID 717130, 7 pages, 2011.

[35] S. Brunelli and P. Rovere-Querini, "The immune system and the repair of skeletal muscle," *Pharmacological Research*, vol. 58, no. 2, pp. 117–121, 2008.

[36] B. Chazaud, M. Brigitte, H. Yacoub-Youssef et al., "Dual and beneficial roles of macrophages during skeletal muscle regeneration," *Exercise and Sport Sciences Reviews*, vol. 37, no. 1, pp. 18–22, 2009.

[37] J. G. Tidball and S. A. Villalta, "Regulatory interactions between muscle and the immune system during muscle regeneration," *American Journal of Physiology: Regulatory Integrative and Comparative Physiology*, vol. 298, no. 5, pp. R1173–R1187, 2010.

[38] A. Mauro, "Satellite cell of skeletal muscle fibers," *The Journal of Biophysical and Biochemical Cytology*, vol. 9, pp. 493–495, 1961.

[39] F. Relaix and P. S. Zammit, "Satellite cells are essential for skeletal muscle regeneration: the cell on the edge returns centre stage," *Development*, vol. 139, no. 16, pp. 2845–2856, 2012.

[40] F. S. Tedesco, A. Dellavalle, J. Diaz-Manera, G. Messina, and G. Cossu, "Repairing skeletal muscle: regenerative potential of skeletal muscle stem cells," *Journal of Clinical Investigation*, vol. 120, no. 1, pp. 11–19, 2010.

[41] G. Paulsen, R. Crameri, H. B. Benestad et al., "Time course of leukocyte accumulation in human muscle after eccentric exercise," *Medicine and Science in Sports and Exercise*, vol. 42, no. 1, pp. 75–85, 2010.

[42] K. Zerria, E. Jerbi, S. Hammami et al., "Recombinant integrin CD11b A-domain blocks polymorphonuclear cells recruitment and protects against skeletal muscle inflammatory injury in the rat," *Immunology*, vol. 119, no. 4, pp. 431–440, 2006.

[43] C. F. P. Teixeira, S. R. Zamunér, J. P. Zuliani et al., "Neutrophils do not contribute to local tissue damage, but play a key role in skeletal muscle regeneration, in mice injected with Bothrops asper snake venom," *Muscle and Nerve*, vol. 28, no. 4, pp. 449–459, 2003.

[44] N. Dumont, P. Bouchard, and J. Frenette, "Neutrophil-induced skeletal muscle damage: a calculated and controlled response following hindlimb unloading and reloading," *American Journal of Physiology: Regulatory Integrative and Comparative Physiology*, vol. 295, no. 6, pp. R1831–R1838, 2008.

[45] J. G. Tidball, E. Berchenko, and J. Frenette, "Macrophage invasion does not contribute to muscle membrane injury during inflammation," *Journal of Leukocyte Biology*, vol. 65, no. 4, pp. 492–498, 1999.

[46] B. Chazaud, "Macrophages: supportive cells for tissue repair and regeneration," *Immunobiology*, vol. 219, no. 3, pp. 172–178, 2014.

[47] C. Johnson-Léger, M. Aurrand-Lions, and B. A. Imhof, "The parting of the endothelium: miracle, or simply a junctional affair?" *Journal of Cell Science*, vol. 113, part 6, pp. 921–933, 2000.

[48] M. R. Elliott and K. S. Ravichandran, "Clearance of apoptotic cells: implications in health and disease," *Journal of Cell Biology*, vol. 189, no. 7, pp. 1059–1070, 2010.

[49] B.-Z. Qian and J. W. Pollard, "Macrophage diversity enhances tumor progression and metastasis," *Cell*, vol. 141, no. 1, pp. 39–51, 2010.

[50] I. S. Mclennan, "Degenerating and regenerating skeletal muscles contain several subpopulations of macrophages with distinct spatial and temporal distributions," *Journal of Anatomy*, vol. 188, part 1, pp. 17–28, 1996.

[51] B. Chazaud, C. Sonnet, P. Lafuste et al., "Satellite cells attract monocytes and use macrophages as a support to escape apoptosis and enhance muscle growth," *Journal of Cell Biology*, vol. 163, no. 5, pp. 1133–1143, 2003.

[52] M. Summan, G. L. Warren, R. R. Mercer et al., "Macrophages and skeletal muscle regeneration: a clodronate-containing liposome depletion study," *American Journal of Physiology: Regulatory Integrative and Comparative Physiology*, vol. 290, no. 6, pp. R1488–R1495, 2006.

[53] N. Dumont and J. Frenette, "Macrophages protect against muscle atrophy and promote muscle recovery in vivo and in vitro: a mechanism partly dependent on the insulin-like growth factor-1 signaling molecule," *American Journal of Pathology*, vol. 176, no. 5, pp. 2228–2235, 2010.

[54] K. Lolmede, L. Campana, M. Vezzoli et al., "Inflammatory and alternatively activated human macrophages attract vessel-associated stem cells, relying on separate HMGB1- and MMP-9-dependent pathways," *Journal of Leukocyte Biology*, vol. 85, no. 5, pp. 779–787, 2009.

[55] J. G. Tidball, "Interactions between muscle and the immune system during modified musculoskeletal loading," *Clinical*

Orthopaedics and Related Research, no. 403, pp. S100–S109, 2002.

[56] J. E. Heredia, L. Mukundan, F. M. Chen et al., "Type 2 innate signals stimulate fibro/adipogenic progenitors to facilitate muscle regeneration," *Cell*, vol. 153, no. 2, pp. 376–388, 2013.

[57] M. Brigitte, C. Schilte, A. Plonquet et al., "Muscle resident macrophages control the immune cell reaction in a mouse model of notexin-induced myoinjury," *Arthritis and Rheumatism*, vol. 62, no. 1, pp. 268–279, 2010.

[58] G. L. Warren, T. Hulderman, D. Mishra et al., "Chemokine receptor CCR_2 involvement in skeletal muscle regeneration," *FASEB Journal*, vol. 19, no. 3, pp. 413–415, 2005.

[59] C. O. Martinez, M. J. McHale, J. T. Wells et al., "Regulation of skeletal muscle regeneration by CCR_2-activating chemokines is directly related to macrophage recruitment," *American Journal of Physiology: Regulatory Integrative and Comparative Physiology*, vol. 299, no. 3, pp. R832–R842, 2010.

[60] L. Zhang, L. Ran, G. E. Garcia et al., "Chemokine CXCL16 regulates neutrophil and macrophage infiltration into injured muscle, promoting muscle regeneration," *American Journal of Pathology*, vol. 175, no. 6, pp. 2518–2527, 2009.

[61] L. Arnold, A. Henry, F. Poron et al., "Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis," *Journal of Experimental Medicine*, vol. 204, no. 5, pp. 1057–1069, 2007.

[62] E. Rigamonti, T. Touvier, E. Clementi, A. A. Manfredi, S. Brunelli, and P. Rovere-Querini, "Requirement of inducible nitric oxide synthase for skeletal muscle regeneration after acute damage," *Journal of Immunology*, vol. 190, no. 4, pp. 1767–1777, 2013.

[63] M. Saclier, H. Yacoub-Youssef, A. L. Mackey et al., "Differentially activated macrophages orchestrate myogenic precursor cell fate during human skeletal muscle regeneration," *Stem Cells*, vol. 31, no. 2, pp. 384–396, 2013.

[64] D. Ruffell, F. Mourkioti, A. Gambardella et al., "A CREB-C/EBPβ cascade induces M2 macrophage-specific gene expression and promotes muscle injury repair," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 41, pp. 17475–17480, 2009.

[65] E. Perdiguero, P. Sousa-Victor, V. Ruiz-Bonilla et al., "p38/MKP-1-regulated AKT coordinates macrophage transitions and resolution of inflammation during tissue repair," *Journal of Cell Biology*, vol. 195, no. 2, pp. 307–322, 2011.

[66] D. Sag, D. Carling, R. D. Stout, and J. Suttles, "Adenosine 5$\prime$-monophosphate-activated protein kinase promotes macrophage polarization to an anti-inflammatory functional phenotype," *Journal of Immunology*, vol. 181, no. 12, pp. 8633–8641, 2008.

[67] R. Mounier, M. Théret, L. Arnold et al., "AMPKα1 regulates macrophage skewing at the time of resolution of inflammation during skeletal muscle regeneration," *Cell Metabolism*, vol. 18, no. 2, pp. 251–264, 2013.

[68] D. Burzyn, W. Kuswanto, D. Kolodin et al., "A special population of regulatory T cells potentiates muscle repair," *Cell*, vol. 155, no. 6, pp. 1282–1295, 2013.

[69] A. E. H. Emery, "The muscular dystrophies," *The Lancet*, vol. 359, no. 9307, pp. 687–695, 2002.

[70] S. Decary, C. B. Hamida, V. Mouly, J. P. Barbet, F. Hentati, and G. S. Butler-Browne, "Shorter telomeres in dystrophic muscle consistent with extensive regeneration in young children," *Neuromuscular Disorders*, vol. 10, no. 2, pp. 113–120, 2000.

[71] M. Vezzoli, P. Castellani, G. Corna et al., "High-mobility group box 1 release and redox regulation accompany regeneration and remodeling of skeletal muscle," *Antioxidants and Redox Signaling*, vol. 15, no. 8, pp. 2161–2174, 2011.

[72] S. A. Villalta, H. X. Nguyen, B. Deng, T. Gotoh, and J. G. Tidbal, "Shifts in macrophage phenotypes and macrophage competition for arginine metabolism affect the severity of muscle pathology in muscular dystrophy," *Human Molecular Genetics*, vol. 18, no. 3, pp. 482–496, 2009.

[73] S. Hodgetts, H. Radley, M. Davies, and M. D. Grounds, "Reduced necrosis of dystrophic muscle by depletion of host neutrophils, or blocking TNFα function with Etanercept in mdx mice," *Neuromuscular Disorders*, vol. 16, no. 9-10, pp. 591–602, 2006.

[74] M. D. Grounds and J. Torrisi, "Anti-TNFα (Remicade) therapy protects dystrophic skeletal muscle from necrosis," *FASEB Journal*, vol. 18, no. 6, pp. 676–682, 2004.

[75] S. A. Villalta, B. Deng, C. Rinaldi, M. Wehling-Henricks, and J. G. Tidball, "IFN-γ promotes muscle damage in the mdx mouse model of duchenne muscular dystrophy by suppressing M2 macrophage activation and inhibiting muscle cell proliferation," *Journal of Immunology*, vol. 187, no. 10, pp. 5419–5428, 2011.

[76] K. Ohlendieck and K. P. Campbell, "Dystrophin-associated proteins are greatly reduced in skeletal muscle from mdx mice," *Journal of Cell Biology*, vol. 115, no. 6, pp. 1685–1694, 1991.

[77] B. Weller, G. Karpati, and S. Carpenter, "Dystrophin-deficient mdx muscle fibers are preferentially vulnerable to necrosis induced by experimental lengthening contractions," *Journal of the Neurological Sciences*, vol. 100, no. 1-2, pp. 9–13, 1990.

[78] J. E. Brenman, D. S. Chao, H. Xia, K. Aldape, and D. S. Bredt, "Nitric oxide synthase complexed with dystrophin and absent from skeletal muscle sarcolemma in Duchenne muscular dystrophy," *Cell*, vol. 82, no. 5, pp. 743–752, 1995.

[79] W.-J. Chang, S. T. Iannaccone, K. S. Lau et al., "Neuronal nitric oxide synthase and dystrophin-deficient muscular dystrophy," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 17, pp. 9142–9147, 1996.

[80] R. H. Crosbie, R. Barresi, and K. P. Campbell, "Loss of sarcolemma nNOS in sarcoglycan-deficient muscle," *FASEB Journal*, vol. 16, no. 13, pp. 1786–1791, 2002.

[81] Y. Sunada, H. Ohi, A. Hase et al., "Transgenic mice expressing mutant caveolin-3 show severe myopathy associated with incrased nNOS activity," *Human Molecular Genetics*, vol. 10, no. 3, pp. 173–178, 2001.

[82] M. Wehling, M. J. Spencer, and J. G. Tidball, "A nitric oxide synthase transgene ameliorates muscular dystrophy in mdx mice," *Journal of Cell Biology*, vol. 155, no. 1, pp. 123–131, 2001.

[83] M. Wehling-Henricks and J. G. Tidball, "Neuronal nitric oxide synthase-rescue of dystrophin/utrophin double knockout mice does not require nNOS localization to the cell membrane," *PLoS ONE*, vol. 6, no. 10, Article ID e25071, 2011.

[84] S. A. Villalta, C. Rinaldi, B. Deng, G. Liu, B. Fedor, and J. G. Tidball, "Interleukin-10 reduces the pathology of mdx muscular dystrophy by deactivating M1 macrophages and modulating macrophage phenotype," *Human Molecular Genetics*, vol. 20, no. 4, Article ID ddq523, pp. 790–805, 2011.

[85] P. Zordan, C. Sciorati, L. Campana et al., "The nitric oxide-donor molsidomine modulates the innate inflammatory response in a mouse model of muscular dystrophy," *European Journal of Pharmacology*, vol. 715, no. 1–3, pp. 296–303, 2013.

[86] P. Rovere-Querini, E. Clementi, and S. Brunelli, "Nitric oxide and muscle repair: multiple actions converging on therapeutic efficacy," *European Journal of Pharmacology*, 2013.

[87] M. C. Dalakas and K. Sivakumar, "The immunopathologic and inflammatory differences between dermatomyositis, polymyositis and sporadic inclusion body myositis," *Current Opinion in Neurology*, vol. 9, no. 3, pp. 235–239, 1996.

[88] A. Ghirardello, S. Zampieri, E. Tarricone, L. Iaccarino, L. Gorza, and A. Doria, "Cutting edge issues in polymyositis," *Clinical Reviews in Allergy and Immunology*, vol. 41, no. 2, pp. 179–189, 2011.

[89] P. Venalis and I. E. Lundberg, "Immune mechanisms in polymyositis and dermatomyositis and potential targets for therapy," *Rheumatology*, vol. 53, no. 3, pp. 397–405, 2014.

[90] R. Hohlfeld, A. G. Engel, N. Goebels, and L. Behrens, "Cellular immune mechanisms in inflammatory myopathies," *Current Opinion in Rheumatology*, vol. 9, no. 6, pp. 520–526, 1997.

[91] K. M. Rostasy, J. Schmidt, E. Bahn et al., "Distinct inflammatory properties of late-activated macrophages in inflammatory myopathies," *Acta Myologica*, vol. 27, pp. 49–53, 2008.

[92] J. Reimann, S. Schnell, S. Schwartz, K. Kappes-Horn, R. Dodel, and M. Bacher, "Macrophage migration inhibitory factor in normal human skeletal muscle and inflammatory myopathies," *Journal of Neuropathology and Experimental Neurology*, vol. 69, no. 6, pp. 654–662, 2010.

[93] S. Khan and L. Christopher-Stine, "Polymyositis, dermatomyositis, and autoimmune necrotizing myopathy: clinical features," *Rheumatic Disease Clinics of North America*, vol. 37, no. 2, pp. 143–158, 2011.

[94] C. Preuße, H. H. Goebel, J. Held et al., "Immune-mediated necrotizing myopathy is characterized by a specific Th1-M1 polarized immune profile," *The American Journal of Pathology*, vol. 181, no. 6, pp. 2161–2171, 2012.