Investigation of wild-type and mycolactone-negative mutant *Mycobacterium ulcerans* on skeletal muscle: IGF-1 protects against mycolactone-induced muscle catabolism

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Dufresne SS, Frenette J. Investigation of wild-type and mycolactone-negative mutant *Mycobacterium ulcerans* on skeletal muscle: IGF-1 protects against mycolactone-induced muscle catabolism. *Am J Physiol Regul Integr Comp Physiol* 304: R753–R762, 2013. First published March 13, 2013; doi:10.1152/ajpregu.00587.2012.—Buruli ulcer (BU), which is caused by *Mycobacterium ulcerans* (MU), is an endemic and neglected tropical disease that affects mostly subtropical zones and is closely related to tuberculosis and leprosy (38, 47). It is widely distributed in more than 30 countries, and the rate of morbidity may surpass tuberculosis and leprosy in some endemic regions of Africa (2, 49). Each year, between 5,000 and 6,000 new cases of BU are reported, and more than 50% of whom are children under 15 years of age (10, 50). Recent animal studies have shown that BU spreads well beyond the skin and significantly affects the underlying tissues, including skeletal muscles. Skeletal muscle under infected skin is also subject to serious dysfunctions and contractures. The goal of this study was to investigate the effects of an infection with the wild-type *M. ulcerans* (WT-MU) or the mycolactone-negative *Mycobacterium ulcerans* (Mneg-MU) mutant strains on myotubes or fully differentiated skeletal muscles. WT-MU infection decreased by 22% and 29% the maximal muscle force at days 7 and 42 postinfection, respectively, while Mneg-MU induced no decrease at day 7 postinfection and a small but significant 13% decrease in muscle force at day 42. A 13.2-fold and 4.3-fold increase in neutrophil and macrophage concentrations, respectively, was observed on day 42 following the injection of WT-MU. However, the increases in neutrophil and macrophage concentrations were 2.4-fold and 5.5-fold in Mneg-MU. Myoblast proliferation decreased by 20%, myotube diameter by 45%, MyHC levels by 32%, while MuRF1 levels increased by 22.8% when C2C12 cells and WT-MU were cocultured for 48 h at a multiplicity of infection of 5:1. In contrast, Mneg-MU had no significant effect. Interestingly, the addition of 1,000 ng/ml of IGF-1 to the WT-MU/C2C12 coculture significantly improved all of these biological parameters. The present investigation clearly established that muscle dysfunction and chronic inflammation in the presence of WT-MU are largely caused by the release of mycolactone, and the addition of recombinant IGF-1 was sufficient to alleviate some of the antiproliferative and atrophic effects of mycolactone.

Mycolactone, a macrolide toxin with highly destructive capacities, is the deleterious agent of MU. It can cause skin necrosis, cell cycle arrest, immunosuppression, cell death by apoptosis, and necrosis (19, 23, 36, 48). Mycolactone-negative MU mutants are reportedly nonvirulent and noncytotoxic for subcutaneous tissues and leukocytes (1, 8, 36). Mycolactone diffuses passively through infected tissues and can rapidly penetrate cells. It enters the cytoplasm but not the nuclear membrane (45). Once inside the cell, mycolactone blocks the expression of several cytokines and chemokines in leukocytes (7, 24, 42). Recent evidence on human monocytes suggests that the mechanism by which mycolactone modulates protein synthesis inhibits the translation of mRNA into a specific subset of proteins (42). The injection of mycolactone into soleus muscles induces an important, persistent loss in muscle force. Mycolactone hijacks some key components of the muscle growth and repair processes and, as a result, impairs muscle regeneration (26).

Akt1, or PKB, is one of the most important signaling pathways for protein synthesis and muscle growth. IGF-1 activates Akt1 and acts as a key regulator of both atrophy and hypertrophy (35). The phosphorylation of Akt1 by the IGF-1 receptor blocks muscle protein breakdown by downregulating ubiquitinite E3 ligase MAFbx/atrogin-1 and muscle ring finger protein 1 (MuRF1) through the phosphorylation (inactivation) of the Forkhead Box O (FOXO) transcription factor (40). Akt1 is also well known for its ability to stimulate the mammalian target of rapamycin (mTOR), which, in turn, promotes protein synthesis by phosphorylating p70-S6 kinase 1 and eukaryotic initiation factor 4E binding protein-1 (3a). Since mycolactone is a macrolide molecule that purportedly acts independently of the Forkhead Box O (FOXO) transcription factor (40). Akt1 is also well known for its ability to stimulate the mammalian target of rapamycin (mTOR), which, in turn, promotes protein synthesis by phosphorylating p70-S6 kinase 1 and eukaryotic initiation factor 4E binding protein-1 (3a). Since mycolactone is a macrolide molecule that purportedly acts independently of mTOR and that this signaling pathway is a potential effector of IGF-1, using IGF-1 to counter the catabolic effects of mycolactone is worth investigating (40).

The first objective of the present study was to compare and characterize the effect of wild-type *Mycobacterium ulcerans* (WT-MU) and a mycolactone-negative mutant *M. ulcerans* (Mneg-MU) strain on fully differentiated skeletal muscles and on cultured myoblasts and myotubes. The second objective was to determine whether the addition of exogenous IGF-1 would rescue myoblast proliferation and prevent mycolactone-induced myotube atrophy. The hypotheses were that only WT-MU would rescue myoblast proliferation and prevent mycolactone-induced myotube atrophy. The hypotheses were that only WT-MU would influence differentiated skeletal muscle/cultured muscular cells and that IGF-1 would protect myoblasts and myotubes against mycolactone. Our results confirmed that WT-MU and, to a much lesser extent, Mneg-MU decreased muscle force and increased leukocyte accumulation in brachial...
biceps muscles, a subcutaneous muscle near the site of infection. Moreover, WT-MU, but not M<sup>es-MU</sup>, inhibited myoblast proliferation and induced myotube atrophy, mainly through the activation of MuRF-1. The addition of exogenous IGF-1 rescued myoblast proliferation and myotube atrophy, downregulated MuRF-1 expression, and preserved the content in myosin heavy chain (MyHC), the key protein of the contractile apparatus.

**MATERIALS AND METHODS**

**Animals.** Ninety-six male C57BL/6 mice weighing 20–22 g (Charles River Laboratories, Senneville, QC, Canada) were housed one per cage in our animal facility. Food and water were provided ad libitum. All animal procedures were approved by the Université Laval Research Center Animal Care and Use Committee, according to Canadian Council on Animal Care guidelines.

**Mycobacterium ulcerans/mycolactone.** WT-MU was originally isolated from a human ulcer in 2005 and was identified by RT-PCR using the specific insertion sequence IS2404 and by Zielh-Neelsen staining (25–28). M<sup>es-MU</sup> was a spontaneous mutant isolated from nonpigmented colonies of MU 1615, a strain from Malaysia that produces mycolactone A/B (34). M<sup>es-MU</sup> was kindly given by Dr. Pamela L. C. Small (University of Tennessee, Knoxville, TN). The two strains were grown for 6–8 wk on Loewenstein-Jensen agar medium at 30°C in a low-oxygen environment containing 5% CO<sub>2</sub>. Purified and synthetic mycolactone A/B was graciously provided by Dr. Yoshito Kishi (Harvard University).

**Injection procedures.** WT-MU and M<sup>es-MU</sup> were suspended and homogenized in ID broth solution [purified water (1 liter), potassium chloride (7.5 g), calcium chloride (0.5 g), tricine glycine (0.895 g), and polysorbate 80 (0.025%)] (BD Pharmingen, San Jose, CA) using a syringe needle, and total counts were determined using a Petroff-Hauser chamber and a microscope at ×400 magnification (46). The mice were anesthetized with isoflurane. WT-MU and M<sup>es-MU</sup> were subcutaneously injected at a concentration of 1 × 10<sup>6</sup> acid-fast bacilli (30 μl; AFB) in proximity to the right brachial biceps [proximate-infected biceps (PBI)]. The mice were killed on days 3, 7, or 2 postinfection. Mice uninfected or subcutaneously injected with ID broth solution (30 μl) in proximity of the right biceps were used as controls.

**Isometric contractile properties and tissue freezing.** The mice received buprenorphine as an analgesic (0.1 mg/kg ip) at least 15 min after being anesthetized with pentobarbital sodium (50 mg/kg ip) (18). The right brachial biceps were dissected and incubated in a buffered physiological salt solution (Krebs-Ringer), as previously described (16, 18, 41). Time-to-peak twitch tension (TPT; ms), half-relaxation time (1/2 RT, ms), twitch tension (Pt, g), maximum tetanic tension (P<sub>0</sub>, g), and maximum specific tetanic tension (sP<sub>0</sub>, N/cm<sup>2</sup>) values were obtained using a 305B-LR dual-mode lever arm system controlled by dynamic muscle control and data acquisition software (Aurora Scientific, Aurora, ON, Canada). The cross-sectional areas of the brachial biceps were estimated by dividing the wet weight by the length of the muscle. The sections were then immersed in methylene blue (Sigma-Aldrich). Three images of each muscle section were randomly acquired (0.2% gelatin, 3% BSA, 2% horse serum, and 0.02% sodium azide). The sections were immunolabeled for 2 h at room temperature with rat anti-mouse F4/80 (1:100; Serotec, Oxford, UK) or Ly-6G and Ly-6C (Gr-1) antibodies (1:300; BD Pharmingen) to identify macrophages and neutrophils, respectively. Marked cells were counted at ×400 magnification, and the total areas of the sections were determined and multiplied by the thicknesses to express the number of each cell type per cubic millimeter. The concentrations of inflammatory cells were measured in duplicate in two midbelly sections of right biceps muscles.

**Myofiber cross-sectional area.** Muscle tissue (10 μm thick) of the biceps was stained with H&E (Sigma-Aldrich). Three images of each muscle section were randomly acquired (×200 magnification), and 150 fibers per section were analyzed. Myofiber cross-sectional areas were calculated using ImageJ software (version 1.43; NIH, Bethesda, MD).

**Cell viability rate.** To determine noncytotoxic conditions, myotubes and myoblasts were incubated with WT MU, M<sup>es-MU</sup>, 10 ng/ml of mycolactone, and 1,000 ng of IGF-1 during 48 h. Cells were harvested and centrifuged at 500 g for 3 min, washed once with PBS, and resuspended in 200 μl media culture without serum on ice. The viability rate was quantified by flow cytometry using FITC annexin V/propidium iodide (PI) labeling of the myoblasts and myocyte (apoptosis detection kit TACS by flow cytometry; Trevigen, Gaithersburg, MD). Annexin V labeled with fluorochrome can identify apoptotic cells by binding to phospholipid phosphatidylserine exposed on the outer leaflet of the plasma membrane, while PI is a standard flow cytometric viability probe that discriminates between viable and nonviable cells. Thus, cells positive for FITC Annexin V and negative for PI undergo apoptosis. Cells positive for FITC Annexin V and PI are either at the end stage of apoptosis, are undergoing necrosis, or are already dead. Cells staining negative for FITC Annexin V and PI are alive. Flow cytometry was performed on a FACSCanto flow cytometer (BD Biosciences), and data were analyzed using FlowJo software.

**Zielh-Neelsen staining.** To localize MU in tissue, transversal sections (10 μm thick) of biceps muscles and skin were cut at −20°C (CM1850 cryostat; Leica Microsystems, Germany) and adhered to Snowcoat X-tra slides (Surpath, Richmond, IL). Tissue sections were treated consecutively with hot carbol fuschin solution for 10 min, tap water for 1 min, 20% sulfuric acid for 1 min, and tap water for 1 min. Tissue sections were then immersed in methylene blue during 1 min, rinsed with tap water and mounted. AFB stain pink, straight, or slightly curved and rod-shaped, while some have a beaded appearance.

**C2C12 myoblast proliferation.** C2C12 myoblasts were cultured in DMEM (HyClone) supplemented with 10% FBS and were seeded in DMEM supplemented with 1% FBS in 96-well plates at a density of 3,000 or 8,000 myoblasts/well for 24- and 48-h proliferation assays. The numbers of WT-MU and M<sup>es-MU</sup> were estimated using a Petrof-Hauser counting chamber. The MU were added to the cultured myoblasts at multiplicities of infection (MOI) of 1:1, 2:1, or 5:1 (MU/myoblast). The MU/myoblast cocultures were then incubated at 30°C for 24 or 48 h, and myoblast proliferation was determined using the tetrazolium colorimetric method (CellTiter 96 Aqueous; Promega, Madison, WI) and compared with standard curve. In a second set of experiments, C2C12 myoblasts were exposed to the same numbers of WT-MU or to 10 ng/ml of pure mycolactone. Thereafter, the WT-MU/myoblast cocultures and myoblasts that had been exposed to mycolactone were treated with various concentrations of recombinant IGF-1 (0, 100, 200, 400, 500, or 1,000 ng/ml) (R&D Systems, Minneapolis, MN) during 24 and 48 h.

**C2C12 myotube diameter analysis.** Confluent myoblasts in 6-well plates (300,000 cells/well) were incubated in DMEM supplemented with 1% FBS for 1 wk to differentiate them into myotubes. WT-MU and M<sup>es-MU</sup> at MOI of 1:1, 2:1, or 5:1 (MU/myoblast) based on the
initial number of myoblasts or mycolactone (10 ng/ml) were then added to the myotubes for 24 and 48 h. Myotube diameters were measured using a light microscope (Nikon, Japan) at ×100 magnification (16). Three sites in each well were blindly identified and were observed throughout the experiment. Myotube diameters were quantified using ImageJ software (version 1.43; National Institutes of Health, Bethesda, MD). Two or three measurements were performed per myotube and were averaged to give a single value. The diameters of 150 to 200 myotubes per well were measured.

**Western blots.** WT-MU (MOI of 1/1 and 5/1), M<sup>reg</sup>-MU (MOI of 1/1 and 5/1), mycolactone (10 ng/ml), IGF-1 (1,000 ng/ml), and various combinations were then added to the myotubes for 48 h. The treated myotubes were washed with PBS, incubated for 30 min at 4°C in lysis buffer containing P8340 protease inhibitor cocktail (1 μM/l; Sigma), and centrifuged for 10 min at 10,000 g. The protein contents of the homogenates were measured using BCA protein assay kits (EMD Chemical, Darmstadt, Germany). Absorbance was measured at 560 nm and was compared with a standard curve. For the Western blot analyses, 40 μg of protein was separated on 9% SDS-polyacrylamide gels, and the protein bands were transferred to polyvinylidene difluoride membranes. The membranes were blocked for 1 h in 5% skim milk and were then incubated overnight at 4°C with anti-MyHC (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Akt1 (1:200; Santa Cruz Biotechnology), anti-phosphorylated-Akt1, (1:200; EMD Millipore, Temecula, CA), anti-atrogin-1 (1:500; ECM Biosciences, Versailles, KY) or anti-MuRF-1 antibody (1:1,000; Santa Cruz Biotechnology). The membranes were rinsed and were incubated for 1 h with goat anti-rabbit (for MyHC, atrogin-1, and phosphorylated-Akt1, 1:10 000; Santa Cruz Biotechnology), or bovine anti-goat (for MuRF-1 and Akt1, 1:10,000; Santa Cruz Biotechnology) horseradish peroxidase-conjugated secondary antibodies diluted in 5% skim milk. The bands were revealed using the ECL-Plus chemiluminescent detection system (PerkinElmer, Waltham, MA). Images of the membranes were acquired, scanned, and analyzed using Quantity One software (version 4.6.6; Bio-Rad Laboratories, Hercules, CA). Because IGF-1 and mycolactone modulate the expression of several muscle proteins frequently used as internal controls (actin, desmin, histone 3, and GAPDH), we stained Western blots with Ponceau Red software, version 3). When a significant variation among the experimental groups was significant (InStat software, version 3). A posteriori test was performed (Tukey’s protected least-significant differences test) to determine whether there were any specific differences. The level of significance was set at P < 0.05.

**RESULTS**

**WT-MU and, to a much lesser extent, M<sup>reg</sup>-MU are found in periphery of skeletal muscle, and induced leukocyte accumulation and loss of muscle function.** Ziehl-Neelsen staining showed positive labeling for AFB after 42 days in the skin of mice injected with WT-MU or M<sup>reg</sup>-MU (Fig. 1A). PIB muscles were also positive (3/8 biceps muscles) for AFB in mice injected with MT-MU at 42 days postinfection. PIB muscles that were positive for AFB exhibited more important muscle necrosis and damage. All PIB muscles with M<sup>reg</sup>-MU were negative for AFB. Furthermore, WT-MU increased neutrophil concentration by 15- and 13-fold at days 7 and 42 postinfection, respectively (Fig. 1, B and C). WT-MU also increased the macrophage concentration by 4.5- and 4.3-fold at days 7 and 42 postinfection. Although the numbers of neutrophils and macrophages were significantly higher in PIB muscles WT-MU, M<sup>reg</sup>-MU only increased the numbers of neutrophils and macrophages by 5.6- and 2.5-fold, respectively, at day 42 postinfection compared with control values (Fig. 1, B and D). No differences were observed in all experimental groups at day 3 postinfection.

To assess the functional impact of WT-MU and M<sup>reg</sup>-MU, control and PIB muscles were isolated and electrically stimulated to measure their contractile properties. Muscle force production was affected by WT-MU at day 7 postinfection, as indicated by the Pt, P0, and sP0 values that decreased by 18%, 22%, and 25%, respectively. On the other hand, M<sup>reg</sup>-MU had no effect on muscle contractility at day 7 postinfection (Fig. 2). At day 42 after WT-MU infection, the Pt, P0, and sP0 values decreased by 29%, 29%, and 31%, respectively. These results are significantly lower than at day 7 postinfection. Interestingly, M<sup>reg</sup>-MU significantly decreased the Pt and P0 values by 14.4% and 13.9%, respectively, by day 42 postinfection, indicating that the presence of MU alone without mycolactone secretion can negatively affect muscle function. No significant differences with respect to TPT and RT V/ were observed in all groups. Consistent with the loss of muscle force and function in WT-MU, the myofiber cross-sectional area was also decreased by 28% relative to control after 42 days postinfection (Fig. 3). No differences in myofiber cross-sectional area were found in M<sup>reg</sup>-MU.

**WT-MU, but not M<sup>reg</sup>-MU, inhibits myoblast proliferation and induces myotube atrophy.** First, we determined by flow cytometry that 10 ng/ml was very close to the maximum dose of mycolactone that did not induce significant apoptosis and necrosis of myotubes/myoblasts. Indeed, C2C12 myotubes or myoblasts exposed to 10 ng/ml of mycolactone during 48 h had a viability rate of 99 ± 0.5% (Fig. 4). Treatment with 50 ng/ml of mycolactone decreased the viability rate at 52.5% and at 41.9% when the concentration is 100 ng/ml. Cells incubated with MOI of 5/1 of WT-MU and M<sup>reg</sup>-MU are still alive. We then compared and characterized the effect of WT-MU and M<sup>reg</sup>-MU on muscle cell proliferation and atrophy. To do so, myoblasts and myotubes were exposed to four different MOIs of WT-MU and M<sup>reg</sup>-MU or a combination of M<sup>reg</sup>-MU and 10 ng/ml of mycolactone. A MU/myoblast MOI of 1:1 had no significant impact on myoblast proliferation (Fig. 5). However, a WT-MU MOI of 2:1 inhibited myoblast proliferation by ~20%. The inhibitory effect of WT-MU on myoblast proliferation reached 40% at MOIs of 5:1 compared with control myoblasts after 24 and 48 h. M<sup>reg</sup>-MU had no effect on myoblast proliferation after 24 and 48 h, regardless of the MOI. Moreover, the combination of M<sup>reg</sup>-MU and 10 ng/ml of mycolactone decreased myoblast proliferation by more than 50% after 24 and 48 h (Fig. 5). In regard to myotube atrophy, a WT-MU MOI of 1:1 reduced by 19% and 13% myotube diameters after 24 and 48 h, respectively (Fig. 6). Moreover, WT-MU MOIs of 2:1 and 5:1 induced 25–30% and 30–45% decreases in myotube diameter after 24 and 48 h, respectively.

**The addition of exogenous IGF-1 partially rescues the inhibitory effects of WT-MU and mycolactone on myoblast proliferation and myotube atrophy.** Exogenous IGF-1 was added to reverse the inhibitory effect of WT-MU and mycolactone on myoblast proliferation and myotube growth. Proliferation decreased by 45% when the myoblasts were exposed to 10 ng/ml of mycolactone for 24 h. However, IGF-1 (1,000 ng/ml) limited the mycolactone-induced decrease in myoblast proliferation to 16% after 24 h. Mycolactone caused a 66% decrease in myoblast proliferation after 48 h, while the addition of
IGF-1 (1,000 ng/ml) significantly reduced the decrease to 45% relative to control values after 48 h (Fig. 7A). Consistent with the results obtained with mycolactone, the addition of IGF-1 (500–1,000 ng/ml) was sufficient to partially rescue the proliferation of myoblasts cocultured with WT-MU for 24 or 48 h (Fig. 7B). Since myotube atrophy correlates with myofibrillar loss and ubiquitin E3 ligase activation, AKT-1, pAKT-1, atrogin-1, MyHC, and MuRF-1 levels were quantified in the presence of WT-MU, M*neg*-MU, mycolactone, and/or IGF-1. When myotubes were cocultured with WT-MU at a MOI of 5:1, MyHC levels decreased by 32%, while MuRF-1 levels increased by 22% relative to the control values (Fig. 8, A and B). Levels of atrogin-1, AKT-1, and pAKT-1 did not change in this coculture (Fig. 8, C–E). IGF-1 totally prevented the decrease in MyHC levels and reduced MuRF-1 expression to a similar level than control. The M*neg*-MU/myotube coculture

Fig. 1. Histological and immunological analyses from mice infected with Mycobacterium ulcerans. A: biceps muscles and skin from control and sham (CTR/sham), WT-MU, and M*neg*-MU were stained with Ziehl-Neelsen after 42 days postinjection. Ziehl-Neelsen staining showed the presence of M*neg*-MU and WT-MU infection in skin, but only WT-MU was detected in the periphery of some biceps muscles (arrows). B: biceps muscles from control and sham (CTR/sham), WT-MU, and M*neg*-MU were immunolabeled for neutrophils and macrophages. The concentrations of neutrophils (C) and macrophages (D) in biceps muscles were determined at days 3, 7, and 42 postinfection. All data are expressed as means ± SE; n = five independent experiments per group. *Significantly different from CTR, P ≤ 0.05. #Significantly different from M*neg*-MU, P ≤ 0.05. Scale bar = 100 μm.
WT-MU is much more cytotoxic for myoblasts, myotubes, and fully differentiated skeletal muscles than M\textsuperscript{neg}\textsuperscript{e}-MU, indicating that the major culprit involved in muscle damage/dysfunction is mycolactone. However, M\textsuperscript{neg}\textsuperscript{e}-MU still induced a moderate, but significant, loss in muscle force production. Likewise, histological observations showed that M\textsuperscript{neg}\textsuperscript{e}-MU caused significant accumulations of neutrophils and macrophages in the infected muscles, albeit to a much lesser extent than WT-MU. Consistent with these results, Oliveira et al. (36) reported that a nonvirulent MU strain caused chronic leukocyte infiltration in subcutaneous tissues at day 60 postinfection. Since M\textsuperscript{neg}\textsuperscript{e}-MU is reportedly harmless for biological tissues, the presence of neutrophils and macrophages in chronically infected tissues may be responsible for the muscle dysfunction observed on day 42 postinfection (1, 19). Neutrophils are likely responsible for the cell damage, the perpetuation of the pathogenic process, and the inflammation seen in chronically infected tissues (30). The persistence of neutrophils in skeletal muscle infected with MU is consistent with previous results showing that macrophage inflammatory protein-2, a chemokine important for neutrophil recruitment, is continuously produced by macrophages infected with WT-MU (45). Importantly, the excessive release of proteases and reactive oxygen species by neutrophils may be detrimental if uncontrolled or unrepressed (12). For example, the infiltration of neutrophils in a sterile and nonnecrotic condition, such as in the hindlimb unloading and reloading protocol, was not associated with secondary damage to the muscle. However, the addition of lipopolysaccharide in the microenvironment overstimulated neutrophil activity, resulting in the induction of secondary damage to the muscle (13). These results highlight that the stimulatory environment fine-tunes neutrophil activity and suggest that these leukocytes might be harmful in chronic WT-MU infection.

Initially, mycolactone has been associated with reduced leukocyte recruitment in skin and lymph nodes (1, 8, 42).

did not produce any significant changes in MyHC, MuRF-1, atrogin-1, AKT-1, and p-AKT-1 levels (Fig. 8, A–E). However, the addition of mycolactone to the M\textsuperscript{neg}\textsuperscript{e}-MU/myotube coculture induced changes in MyHC and MuRF-1 levels that were similar to those observed with the WT-MU/myotube cocultures.

**DISCUSSION**

BU was originally thought to be associated with an immune system dysfunction (22, 33). We recently showed that BU extends well beyond the skin and significantly affects underlying skeletal muscles (25–28). The present study showed that

**Fig. 2.** Isometric contractile properties of infected biceps muscles. Maximum twitch tension, Pt (A) maximum tetanic force, P\textsubscript{0} (B), and maximum specific tetanic force, sP\textsubscript{0} (C) are shown for control (CTR), ID broth-injected (Sham), WT-MU, and M\textsuperscript{neg}\textsuperscript{e}-MU at days 3, 7, and 42 postinfection. Pt, P\textsubscript{0}, and sP\textsubscript{0} decreased progressively from day 7 through day 42 following the WT-MU infection, while Pt and P\textsubscript{0} decreased significantly by day 42 following the M\textsuperscript{neg}\textsuperscript{e}-MU infection. All data are expressed as means ± SE; n = 8 independent experiments per group. *Significantly different from CTR, P ≤ 0.05. #Significantly different from matched M\textsuperscript{neg}\textsuperscript{e}-MU, P ≤ 0.05.

**Fig. 3.** Physiological fiber cross-sectional areas (PCSA) of biceps muscle are shown for control (CTR), WT-MU, and M\textsuperscript{neg}\textsuperscript{e}-MU at day 42 postinfection. PCSA decreased by 28.02% following the WT-MU infection, while any difference was observed following the M\textsuperscript{neg}\textsuperscript{e}-MU infection. All data are expressed as means ± SE; n = 4 independent experiments per group. *Significantly different from CTR, P ≤ 0.05. #Significantly different from matched M\textsuperscript{neg}\textsuperscript{e}-MU, P ≤ 0.05.
However, cumulative evidence indicates that leukocyte recruitment is clearly tissue specific and that the inhibitory effects of mycolactone on leukocyte recruitment may be different in skeletal muscles (1, 8, 17, 42). For example, L-selectins are highly expressed on activated T cells and are essential for the initial tethering and rolling of circulating lymphocytes. Mycolactone suppresses L-selectin expression, preventing T-cell homing to peripheral lymph nodes (22). However, it appears normal...
that selectins are not required for macrophage invasion since \( \alpha_{4} \) integrin can compensate for selectin-rolling functions in skeletal muscle (17, 29). Moreover, the observation that muscle inflammation is more severe in the presence of WT-MU suggests that mycolactone could recruit leukocytes through an indirect mechanism. Indeed, mycolactone induces cell damage, which, in turn, results in the release of chemoattractant molecules that might recruit leukocytes into necrotic areas of skeletal muscles. Muscle cells, as well as nonmuscle cells such as endothelial cells, adipocytes, mast cells, fibroblasts, and macrophages, are all potential sources of chemoattractants (9). However, their chemotactic activities following MU infections have yet to be defined (1). Together, these results indicate that WT-MU induces mild, chronic tissue inflammation and that the

![Fig. 6. C2C12 myotube atrophy in the presence of Mycobacterium ulcerans. WT-MU, but not M\(^{\text{neg}}\)-MU, induced significant myotube atrophy after 24 and 48 h. Mycolactone (10 ng/ml) also induced significant myotube atrophy. All data are expressed as means ± SE; \( n = 5 \) independent experiments per group. *Significantly different from CTR, \( P \leq 0.05 \). #Significantly different from matched M\(^{\text{neg}}\)-MU, \( P \leq 0.05 \).]

![Fig. 7. IGF-1 partially reverses the negative effect of WT-MU and mycolactone on C2C12 myoblasts. A and B: various concentrations of IGF-1 (0–1,000 ng/ml) were added to the culture medium of C2C12 myoblasts that had previously been treated with 10 ng/ml of mycolactone or WT-MU at an MOI of 5:1. IGF-1 (>200 ng/ml) rescued the proliferation of myoblasts exposed to 10 ng/ml of mycolactone, while >500 ng/ml IGF-1 promoted the proliferation of myoblasts in the presence of WT-MU. Each percentage shows the variation in myoblast proliferation expressed relative to cells treated without IGF-1. All data are expressed as means ± SE; \( n = 5 \) independent experiments per group. *Significantly different from CTR, \( P \leq 0.05 \).]

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The immunosuppressive effect of mycolactone is not sufficient to prevent leukocyte infiltration into skeletal muscle.

Our results also indicated that WT-MU and mycolactone, but not Mneg-MU, decreased myotube diameter and MyHC levels. MyHC is selectively targeted for degradation following cachectic syndromes, muscle disuse, and dystrophy (5, 6, 14). The calcium-dependent cysteine proteases, calpains, are well known for their important role in the initiation of the proteolytic process (32, 43). Interestingly, it has recently been shown that mycolactone mobilizes and partially depletes the intracellular calcium store in T cells in vitro (4). This observation provides support for the possibility that mycolactone may stimulate MyHC degradation through an increase in intracellular calcium mobilization and the activation of calpains. However, while calpains induce the dismantling of complex muscle proteins, the ubiquitin/proteasome pathway, more par-

Fig. 8. Myosin heavy chain (MyHC), muscle ring finger protein-1 (MuRF-1), atrogin-1, Akt-1, and p-Akt-1 levels in myotubes exposed to various conditions in vitro. Expression of MyHC (A), MuRF-1 (B), atrogin-1 (C), Akt-1 (D), and p-Akt-1 (E) relative to control were determined by Western blots from myotubes exposed to WT-MU (1/1 or 5/1), Mneg-MU (1/1 or 5/1), Mneg-MU (5/1), Mneg-MU (5/1) + 10 ng/ml of mycolactone, Mneg-MU (5/1) + 100 ng/ml of IGF-1, Mneg-MU (5/1) + 1,000 ng/ml of IGF-1, or WT-MU (5/1) + 1,000 ng/ml of IGF-1. Protein levels were normalized using the densitometric values determined from Ponceau red-stained blots (lower panel). All data are expressed as means ± SE. All experiments were performed in triplicate. *Significantly different from CTR, P < 0.05. ‡Significantly different from matched Mneg-MU, P < 0.05.
particularly, the ubiquitine E3 ligase MuRF-1 is an important muscle proteolysis system involved in MyHC degradation (43). Indeed, knocking out MuRF-1 prevents the loss of MyHC and protects against muscle atrophy in a variety of muscle-wasting conditions (3, 5, 15). Importantly, WT-MU and mycolactone, but not M\textsuperscript{neg}-MU, stimulated the ubiquitin/proteasome pathway. The present findings also showed that the addition of IGF-1 is sufficient to antagonize the inhibitory effect of mycolactone on myoblast proliferation and myotube atrophy. The binding of IGF-1 to its receptor triggers the PI3K/AKT/mTOR pathway, which stimulates various biological activities such as growth, proliferation, and protein synthesis (31). The activation of the PI3K/AKT/mTOR pathway by IGF-1 also phosphorylates the FOXO transcription factor and, thereby, inhibits the transcriptional rate of the E3 ubiquitin ligases MuRF-1 and MAFbx (20). Our results are, thus, consistent with previous observations suggesting that mycolactone acts independently of PI3K/AKT/mTOR and that the IGF-1 signaling pathway suppresses MuRF-1 activation and prevents the decrease in MyHC levels (11, 24).

**Perspectives and Significance**

Although mycolactone is clearly the culprit of this necrotic and infectious disease, our results indicate that M\textsuperscript{neg}-MU also induces a mild inflammation, which eventually leads to a loss of muscle force. To pinpoint the contribution of all subsets of leukocytes, such as neutrophils, macrophages, eosinophils, and T cells in muscle dysfunction will certainly be worth investigating during the course of BU disease. The observation that IGF-1 also reduces the negative action of mycolactone on myoblast proliferation and myotube atrophy is very interesting and may open new possible interventions for BU. Further studies are required to determine how mycolactone induces muscle atrophy and fibrosis and whether exogenous IGF-1 combined with antibiotics are able to minimize or reverse the extent of muscle injury, atrophy, and dysfunction during the progression of the disease.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

Author contributions: S.S.D. and J.F. conception and design of research; S.S.D. performed experiments; S.S.D. and J.F. analyzed data; S.S.D. and J.F. interpreted results of experiments; S.S.D. and J.F. prepared figures; S.S.D. and J.F. drafted manuscript; S.S.D. and J.F. edited and revised manuscript; S.S.D. and J.F. approved final version of manuscript.

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